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SOME ASPECTS OF REPRODUCTIVE BIOLOGY IN THE FRESH-WATER TRICLAD TURBELLARIAN, CURA FOREMANII [1]

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Apparently alone among the fresh-water triclad turbellarians for which the details of reproduction are known, *Cura foremanii* is capable of sexual reproduction under conditions which prevent the occurrence of copulation between two individuals. This was established for a Rhode Island strain of this species by Anderson (1952a), who isolated individuals on the day of hatching and found that upon reaching sexual maturity each worm deposited numerous cocoons, a large majority of which were usually fertile and produced normal juveniles. Furthermore, it was established that reproduction could continue in this way for long periods without apparent detriment to the viability or reproductive capacity of the strain. In one series of observations, terminated after 20 months, a pedigreed line of worms produced 9 successive generations, each generation represented by one individual isolated as soon as it had hatched and maintained in isolation until its first offspring appeared (Anderson, 1952b and subsequent unpublished observations). As these worms were apparently capable of maintaining reproductive activities indefinitely without opportunity for copulation, and as we had never observed copulation in mass or paired cultures of *Cura*, it was suggested that copulation between individuals might not be a normal prelude to sexual reproduction in this species. Kenk (1935) has reported that *Cura foremanii* lacks a true copulatory bursa, an organ invariably present in copulating species; in *Cura* it is represented only by its stalk, which connects with one of the medial branches of the intestine and forms a genito-intestinal canal. These facts support the suspicion that *Cura* must reproduce without copulation.

In the initial report of the unique reproductive habits of *Cura foremanii*, it was impossible to state whether reproduction in isolated individuals involved fertilization of eggs by spermatozoa from the same worm or parthenogenetic development of unfertilized eggs. Neither parthenogenesis nor self-fertilization had been reported in other fresh-water triclads by previous authors, and no direct evidence favoring either possibility was obtained from our observations.

[1] Referred to in previous publications as *Curtisia foremanii;* best taxonomic account is that of Kenk (1935). The name *Curtisia* (given this worm in 1916 by von Graff, in honor of Prof. W. C. Curtis) is preoccupied; Strand (1942) has proposed the substitution of *Cura* for *Curtisia.*

The apparently simple question of the nature of the reproductive process in this species has proved surprisingly difficult to answer unequivocally. Evidence bearing on the central problem has been sought by attacking several subsidiary questions, such as the following:

1) Is sexual reproduction in isolation characteristic of the species as a whole, or is it limited to the stock with which the original work was done?

2) In sexually mature and reproductive individuals, reared in isolation, are spermatozoa present in the seminal receptacles (the anterior extremities of the ovovitelline ducts, adjacent to the ovaries), as they were reported to be by Curtis (1900) and by Stevens (1904) in animals taken from the wild and from mass cultures?

3) If spermatozoa are found here in isolated individuals, when do they first arrive at the seminal receptacles? Is there any correspondence between the time of first appearance of spermatozoa at the ovaries and the time of deposition of first cocoons by isolated worms just attaining sexual maturity? Or can an individual deposit fertile cocoons before spermatozoa first appear in its seminal receptacles?

4) Do eggs deposited in cocoons by isolated individuals show any evidence of having been penetrated by spermatozoa?

5) Do spermatozoa ever appear in the bursa stalk (genito-intestinal canal) of mature worms taken from mass cultures? That is, is there evidence that what remains of the copulatory bursa is used in copulation in mass cultures? If spermatozoa are found here, how do worms from mass cultures compare in this respect with mature and reproductive individuals reared in isolation?

This paper reports the results of investigations designed to provide answers to these and related questions and discusses the relationship between this information and the major problem of the nature of the reproductive process in *Cura foremanii*.

Materials and Methods

The worms on which these observations have been made constitute several stocks of diverse geographical origins. One stock represents descendants of the specimens collected in 1950 from a stream near Geneva, Rhode Island, in which reproduction in isolation was originally described. Another group of worms originated with several specimens collected in Cascadilla Creek, near Ithaca, New York. A third stock consists of offspring of several mature specimens collected from a stream in the Adirondacks, near Warrensburg, New York, by Neal R. Foster. The fourth strain was purchased from a biological supply house, having been collected in Powder Mill Park, near Rochester, New York.

The methods used in maintaining these stocks are essentially those described earlier (Anderson, 1952a), with some modifications. All worms have for the past few years been kept in a constant-temperature room under continuous illumination. Temperatures have varied over long periods between 21° and 22.5° C.; illumination is provided by two 40-watt fluorescent tubes about four feet above the table on which the cultures are kept. Specimens have been maintained both in mass cultures and in isolation. Each of the isolated worms is kept in about

50 ml. of water in a 4-ounce jar. Small mass cultures (6 to 12 individuals) are kept in larger amounts of water in identical jars; larger mass cultures of several dozen individuals occupy wide-mouth jars containing approximately 200 ml. of water. All culture jars are tightly closed with metal screw caps.

The sources and types of water used for the cultures have been subject to considerable variation through the past several years. When the Rhode Island strain was first transferred to Ithaca, and for some years subsequently, these worms and the locally-collected stocks were kept in water obtained from Cascadilla Creek at the Cornell University fish hatchery. In 1957 all stocks were changed to a "modified tap water" suggested by Loomis and Lenhoff (1956), prepared by adding 50 mg./L each of disodium ethylenediaminetetraacetate and $CaCl_2$ to tap water. For the past few months we have used unmodified Ithaca tap water without apparent unfavorable effects on the cultures.

All worms are fed regularly, once each week. Isolated worms are given bits of fresh mouse-liver, while the mass cultures are fed liver and such other organs as heart, spleen, and kidneys of freshly-killed mice. Food is allowed to remain in the jars for at least several hours and sometimes overnight, after which the remnants are removed and the water changed.

The worms are examined carefully at one- or two-day intervals. For each of the isolated worms, records are kept of the dates of deposition and hatching of all cocoons, and of the number of young produced. Juvenile worms are removed from these cultures as soon as possible after hatching; they are either transferred to mass cultures or set up individually in isolation to maintain and extend selected pedigreed stocks. Following the scheme previously described (Anderson, 1952a), each such isolated juvenile receives a code designation denoting its generation and its ancestry.

For the specific purposes of the present investigation it was necessary to prepare serial sections of mature worms from both mass and isolation cultures, as well as of a series of developing juveniles sacrificed at weekly intervals from the time of hatching to the attainment of sexual maturity. Specimens were anesthetized by placing them in a solution of MS-222,[2] 1:1500 by weight, as suggested by Manner (1957); when relaxed, the worms were flooded with either Zenker-acetic or Helly's fluid. They were kept extended and moderately flattened during fixation by compressing them gently under a coverslip. Following standard procedures, the specimens were imbedded in Tissuemat; serial sections were prepared at 7 μ, mounted, and stained in Harris' hematoxylin. Several freshly deposited cocoons were also fixed, imbedded, sectioned, and similarly stained, to provide material for determination of the nuclear condition of the eggs they contained.

Observations and Discussion

The ability of *Cura foremanii* to carry on active sexual reproduction in isolation is not limited to the Rhode Island strain in which it was originally described. The other three stocks with which we have worked, collected from various localities in central and northern New York, have all proved capable of the same repro-

[2] A supply of this reagent was generously provided by Dr. Perry W. Gilbert. In our experience, exposure of planarians to MS-222 at this concentration almost invariably induces eversion of the proboscis, and the animal relaxes in this condition.

ductive behavior. It therefore seems reasonable to conclude that this kind of reproduction, whatever the details of the process may be, is a general characteristic of the species.

In all mature specimens of which sections have been examined, masses of spermatozoa have been found in the seminal receptacles. Figure 1 is presented as typical. This section is from an adult worm killed in the act of releasing a finished cocoon from its genital atrium. As the figure shows, the ovaries contain developing eggs in various stages of maturity, and the seminal receptacles are occupied by masses of spermatozoa. It is to be emphasized that in view of the anatomical relationships of the reproductive systems in fresh-water triclads, these spermatozoa could have reached the seminal receptacles only by moving anteriorly in the ovovitelline ducts from the genital atrium (see Curtis, 1900, and Kenk, 1935, for anatomical details of the reproductive system of *Cura*). Furthermore, since this individual had been maintained in isolation since the day it hatched, these spermatozoa could have originated only in its own testes and must have been emitted through its penis; spermatozoa are never found wandering about in the mesenchyme but are always restricted to the genital ducts.

All our observations indicate convincingly that in fully mature, actively reproducing specimens of *Cura* the seminal receptacles are invariably packed with spermatozoa; in isolated individuals these can have come only from the testes of the same animal. It is of interest to determine whether in young worms, just reaching sexual maturity, cocoons can be deposited before spermatozoa are produced and reach the seminal receptacles, or whether spermatozoa are already present in the seminal receptacles at the time of deposition of the first cocoon. Table I lists the ages at which 28 individuals of diverse origins, reared in isolation under constant conditions of temperature, light, and feeding, deposited their first cocoons. There is considerable variation, not apparently correlated with strain differences or any other obvious factors; the mean age at sexual maturity for this group of worms is 57.6 ± 2.5 days. Among these specimens, as among large numbers of other isolated individuals for which records have been kept, 40 days is the minimum age at sexual maturity; in all our experience with these worms, no isolated individual has ever deposited its first cocoon before about six weeks from its day of hatching.

The problem now becomes one of determining whether there is any correspondence between this earliest first-cocoon record and the time of arrival of spermatozoa in the seminal receptacles. Our study of this aspect of the problem involved the preparation and study of sections of a series of individuals of known ages, at weekly intervals from one week to seven weeks after the day of emergence from the cocoon. This afforded an opportunity for a general study of the development of both male and female systems, but since the details of such a study are not germane to the present problem only a summary of the principal events will be given. Several individuals of each age were studied, and there was some variation in the stage of development reached by different animals of the same age. Those showing the most rapid rate of development will be described, as they probably represent the group that would have shown earlier-than-average deposition of first cocoons. *At two weeks*, early ovaries and testes are recognizable, containing chiefly gonia and early gametocytes. *At three weeks*, the gonads are

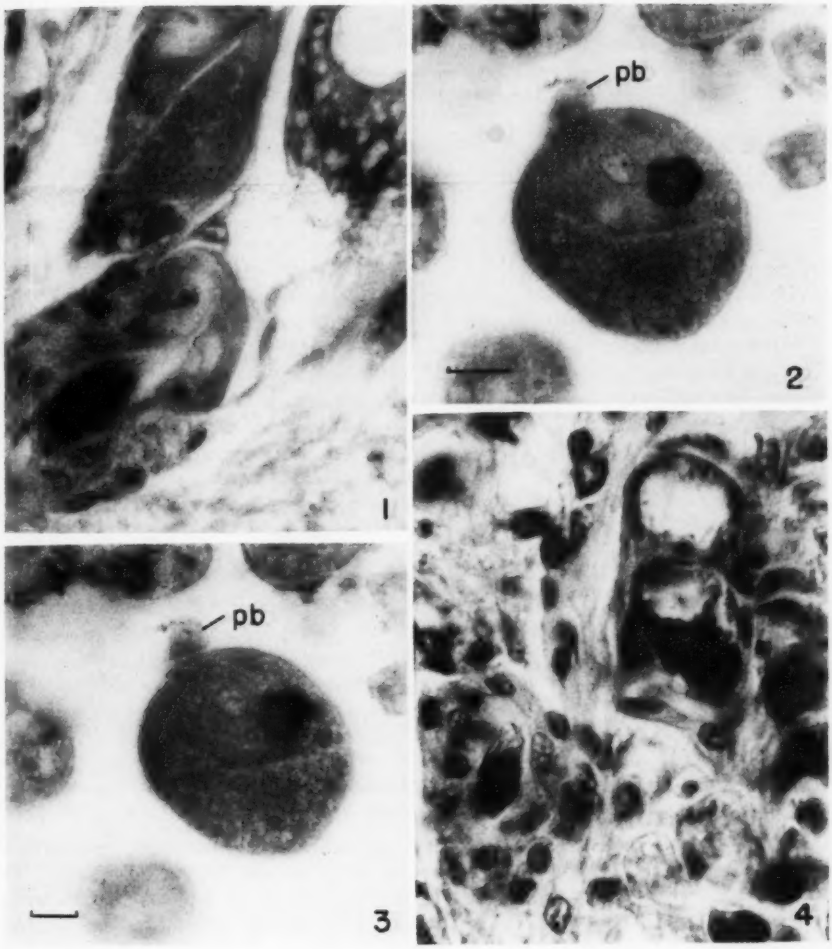

PLATE I

All figures are photomicrographs of sections cut at 7 μ and stained in Harris' hematoxylin. The scales represent 10 microns; that given for Figure 3 indicates magnification for Figures 1 and 4 also.

FIGURE 1. Portion of ovary and seminal receptacle of a mature individual, reared in isolation, fixed at the moment of deposition of a finished cocoon. At upper right, developing eggs in various stages; at lower left, mass of spermatozoa in seminal receptacle.

FIGURE 2. Fertilized egg in a cocoon, surrounded by yolk cells. Second polar body (pb) has been emitted; note vesicular female pronucleus and darkly-stained sperm nucleus.

FIGURE 3. Same section as that shown in Figure 2, at a slightly different focus, showing chromosomes in polar body (pb).

FIGURE 4. Portion of ovary and seminal receptacle of an individual reared in isolation for five weeks from the day of hatching. Note developing eggs in ovary (upper right) and mass of spermatozoa already present in seminal receptacle (lower left) in this animal which is just approaching sexual maturity.

TABLE I

Time required for attainment of sexual maturity at 22.5° C.

Worm	Date hatched	Date of 1st cocoon	Days to 1st cocoon
V9-1a-1a	1/8	2/17	40
T3-9a	1/7	2/17	41
V4-1a	10/9	11/19	42
V4-1b	10/9	11/19	42
*AD-9	10/24	12/10	47
*AD-5	10/24	12/11	48
V9-1a	11/8	12/26	49
T6-8a	1/8	2/28	51
*AD-8	10/24	12/14	51
†W2-1b	2/11	4/4	52
T10-11a	12/28	2/18	52
P8-1a	11/4	12/26	52
V9-12a	12/20	2/11	53
T10-11a-3a	3/20	5/12	53
*AD-3-2b	12/9	1/31	53
V10-1a	11/13	1/8	56
T10-1a	11/1	12/28	57
*AD-9-3a	1/7	3/3	57
†W2-1a	2/11	4/10	58
V6-1a	11/1	12/30	59
*AD-3-2a	12/9	2/7	60
P6-1a-11a	3/31	5/31	61
V4-1b-1a	11/29	2/7	71
V2-1a	11/11	1/22	72
V10-10a	12/20	3/11	81
T10-1a-1a	1/8	4/2	84
P6-1a	11/4	1/29	86
V4-1a-7a	1/2	3/29	86

* Adirondacks strain.
† Rochester strain.
Unmarked individuals are of Rhode Island or Ithaca strains.

larger and contain more advanced stages in gametogenesis; in addition, the earliest signs of development of the posterior genital complex are found at this age. *At four weeks,* large oöcytes are present in the ovaries, and the testes contain what appear to be well-developed spermatozoa. The genital atrium and associated structures are in an advanced state of development, but there are no signs that the ovovitelline ducts and the ductus deferentes are complete. *At five weeks,* the most advanced animal studied showed fully developed ovaries containing eggs well on the way to maturity; the testes were packed with mature spermatozoa; the genital complex posterior to the pharynx was completely formed, and sperm ducts and oviducts were present. Most significantly, at the age of 35 days from hatching, this individual's seminal receptacles were filled with spermatozoa. A portion of a section of this worm, showing some of these features, is presented as Figure 4. All individuals studied *at six and seven weeks* were in similarly advanced stages of sexual maturity, although none had deposited its first cocoon before fixation; in all, the seminal receptacles were filled with spermatozoa. The results of these

studies are consistent with the conclusion that spermatozoa are always present in the seminal receptacles before the individual deposits its first cocoon, and thus that all eggs leaving the ovaries, even those first produced by an individual just reaching sexual maturity, must pass through a cloud of spermatozoa before reaching the genital atrium for inclusion in a cocoon.

Stevens (1904), in her studies of reproduction and early development in wild and mass-cultured specimens of *Cura foremanii* (referred to in her paper as *Planaria simplissima*, following Curtis' identification), reports that the first maturation division of oögenesis takes place within the ovary, and that the secondary oöcytes released into the seminal receptacles are then penetrated by the spermatozoa. The second maturation division occurs as the cocoon is being deposited, or very shortly thereafter, the sperm nucleus remaining quiescent until after the emission of the second polar body. We have now determined that in isolated individuals, just as in the cases reported by Stevens, spermatozoa are always present in the seminal receptacles after the attainment of sexual maturity. The problem remaining is to determine whether in these isolated individuals the secondary oöcytes passing through the seminal receptacles are penetrated by spermatozoa, or whether they remain unfertilized and develop parthenogenetically.

For information on this point we have turned to a study of the contents of freshly deposited cocoons. Each cocoon contains four to six eggs, extremely difficult to locate among the myriads of yolk cells with which they are surrounded. We were unsuccessful in our attempts to identify eggs in squash preparations of fresh cocoons but were able to locate several, in various stages of development, in serial sections. One of these eggs is shown in Figures 2 and 3; these are photographs of the same section, taken at slightly different levels of focus, showing the condition of the egg shortly after the formation of the second polar body. This cell contains, in addition to the vesicular female pronucleus, a densely staining mass which clearly represents the quiescent sperm nucleus (compare with Stevens' figures, 1904). Needless to say, a section such as this is exceptional; but there is no reason for believing that the condition it reveals is anything but typical. On the basis of such evidence we are led to conclude that the eggs of isolated individuals are fertilized, as they pass through the seminal receptacles, by spermatozoa from the testes of the same animal.

Although we may be confident that self-fertilization is the rule in isolated individuals, we have as yet no conclusive evidence as to whether reproduction in mass cultures involves copulation. Kenk (1935), reporting the absence of a copulatory bursa in *Cura*, nevertheless assumed (on what grounds we do not know) that copulation occurs: "In copulation, the relatively small penis is inserted into the posterior part of the bursa stalk 'vagina' of the co-copulant" (p. 82). In our own investigations, three individuals taken from mass cultures were fixed, sectioned, and stained, and their bursal stalks were carefully searched for the presence of spermatozoa. In only one of these were traces of spermatozoa found, but spermatozoa were found also in the bursal stalks of worms reared in isolation, in about the same proportion of cases. This is not a reliable kind of evidence, for two reasons. If copulation does occur between individuals, we do not know how long spermatozoa remain in the bursal stalk "vagina" before migrating into the seminal receptacles; those animals from mass cultures in which the stalks

were empty might simply represent cases in which this migration had been completed. Furthermore, there is no certainty that spermatozoa might not be deposited in the bursal stalk by the penis of the same individual, or might wander into it seeking the openings of the ovovitelline ducts; in the isolated worms, spermatozoa must have reached the stalk in some such manner. Since the stalk forms a genito-intestinal connection, functioning, as Kenk suggests, to carry off "superfluous" sperm to the intestine for digestion, the spermatozoa found in the stalks of our isolated worms may simply be on their way to this fate.

The evidence bearing on the question of copulation in these worms is indirect and inadequate, but it seems a significant fact that the only species in which sexual reproduction is known to occur in the absence of copulation is also a species in which the copulatory bursa has been lost (or never developed at all) and is represented only by a genito-intestinal canal. To settle the question we need, simply, to observe two individuals in the act of copulation; failing this, we need a fertile individual showing some distinct heritable peculiarity of color or form which could be followed through breeding experiments. In our seven years' experience with this species we have observed neither copulation nor a distinctively-marked individual; this is an extremely uniform species. Exposure of large numbers of the worms to x-radiation might produce a mutation which could serve as a genetic marker. Such evidence as we now have, however, indicates the possibility, if not the probability, that *Cura foremanii,* which in isolation can substitute self-fertilization for copulation, may in fact have eliminated copulation as a normal feature of its reproductive processes.

Summary and Conclusions

A careful re-examination of the details of sexual reproduction in isolated individuals of the triclad species *Cura foremanii* has been carried out, designed particularly to determine whether this reproduction involves self-fertilization or simply the parthenogenetic development of unfertilized eggs. The following conclusions have been reached:

1) Without apparent detriment to the species, sexual reproduction can continue for many generations, and evidently indefinitely, under conditions of isolation which prevent the occurrence of copulation between individuals. This is true of four different stocks of the species, of diverse geographical origin.

2) All individuals examined during active reproduction showed masses of spermatozoa occupying the seminal receptacles through which the eggs must pass in moving from the ovaries to the genital atrium.

3) Studies of the development of the reproductive systems in immature isolated worms of known ages reveal that spermatozoa are mature and have already migrated to the seminal receptacles as early as 35 days after the emergence of the individual from its cocoon.

4) Under constant conditions, isolated individuals deposit their first cocoons some time between the 40th and the 86th day after emergence; thus, the eggs in even these first cocoons have always been exposed to spermatozoa in the seminal receptacles.

5) During this exposure to spermatozoa, the eggs are penetrated by them; eggs have been observed in freshly deposited cocoons showing second polar bodies

and vesicular female pronuclei, as well as distinctly staining sperm nuclei. Development of these self-fertilized eggs is presumably normal.

6) It is thus clear that sexual reproduction in isolated individuals of *Cura foremanii* involves self-fertilization and not parthenogenesis.

One tantalizing question remains unanswered: whether copulation ever occurs in *Cura*, even among individuals in mass cultures. This species lacks a copulatory bursa, possessing only the bursa stalk which serves as a genito-intestinal canal. Copulation has not been observed during our experience with this species, and clearly from the standpoint of normal reproduction and development the process is completely superfluous. Breeding experiments with marked individuals might settle the question; until these can be devised, it seems probable that *Cura foremanii* may have dispensed with copulation as a feature of its sexual reproductive processes.

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FORM-STABILITY OF CILIATES IN RELATION TO PRESSURE AND TEMPERATURE[1]

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The first use of hydrostatic pressure as an experimental parameter in biological research is credited to Regnard (1884a, 1884b, 1884c) and Certes (1884), working independently. Both of these early workers were impressed by the variety of living forms that had been recovered by the deep-sea dredging expedition of the *Talisman* in 1882–1883. Regnard, particularly, became interested in the effects of pressure *per se*. He eliminated changes in the gaseous equilibria by applying the pressure directly to the aqueous medium and he studied the effects of pressures ranging up to 1000 atmospheres on a wide variety of small aquatic organisms. In the present connection, he observed that various ciliates, crowded in stagnant water and subjected to pressures of 600 to 1000 atmospheres for 10 minutes, became immobile and distended, and that ciliary movement stopped. Within two hours after decompression many of the organisms seemed to have recovered completely.

Ebbecke (1935, 1936) described the effect of pressure on paramecia. Exposure to pressures of 500 atmospheres for periods of from 10 to 30 minutes mainly effected a change in the shape of the ciliates, *i.e.*, their bodies became more spheroidal. At pressures of from 800 to 1000 atmospheres for periods extending from 5 to 30 minutes, the organisms became spheroidal and many underwent cytolysis. The rounding effects were reversible after a recovery period spanning several days. At 2000 atmospheres there was a drastic rounding of the cells, followed by complete cytolysis of all the organisms.

Hodapp and Luyet (1947) studied the mechanism of death of paramecia subjected to high hydrostatic pressure. They obtained a typical sigmoid curve of the percentage of paramecia killed by pressures varied systematically from 500–1200 atmospheres, each pressure being maintained for two minutes. At 950 atmospheres about 50 per cent of the cells were killed. Hodapp and Luyet also varied the time, the temperature, the rate at which the pressure was increased and decreased, and the age of the cultures, and found that the total compression time and the culture age were most important in relation to lethality. Temperature (between 10°–22° C.) and the rate of pressure increase were reported to have little or no effect upon lethality. In this connection, however, it should be realized that Hodapp and Luyet did not employ a windowed pressure chamber and could not observe the effects until after the organisms were removed from the chamber. Consequently, the compression and decompression effects could not be differentiated.

In 1934, D. E. S. Brown, studying frog muscle, first recognized the interrela-

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tionship between pressure and temperature in biological systems, and an appreciation of this important relationship did much to clarify subsequent investigations in the field. Further insight, particularly with reference to the problem of how pressure induces the solation of protoplasmic gels, was provided by a classification of gelational phenomena, published by Freundlich in 1937. Experiments by Heyman (1935, 1936) had shown that certain gel systems, represented by methyl cellulose, behave oppositely to gelatin. The methyl cellulose type of system displays a volume increment ($+\Delta V$) upon gelation; and Freundlich deduced that such gelations must be endergonic in nature. Then Marsland and Brown (1942) studied the sol-gel equilibria of myosin, methyl cellulose, and gelatin as affected by hydrostatic pressure and temperature. These experiments showed that myosin (*in vitro*), methyl cellulose, and protoplasmic gels, generally, must be placed in a common class of system because all undergo solation as a result of compression and cooling. Gelatin, on the other hand, gelates more firmly with cooling and compression and must represent a different class of system. These workers also emphasized the speed with which protoplasmic sol-gel equilibria may be shifted, particularly in fresh myosin preparations, and they postulated the intervention of an enzyme system (an ATP-ase complex) which likewise is sensitive to pressure and temperature.

The studies of Marsland (1950) demonstrated that the cortical plasmagel of dividing sea urchin eggs reacts to pressure-temperature treatments as do other intracellular gels. Landau *et al.* (1954), performing similar experiments on the plasmagel system of *Amoeba proteus*, came to the same conclusion, namely, that the gel system is weakened by higher pressures and lower temperatures, within the physiological range.

It is apparent, therefore, that pressure-temperature conditions may effect profound changes in cell structure and that such changes are determined, at least partly, by pressure-temperature effects upon the gelational state of the protoplasm. The purpose of the present work, accordingly, is to study the form-stability of two representative ciliates, Blepharisma and Paramecium, under systematically varied conditions of pressure and temperature. Generally, it has been supposed that the characteristic morphology and structural integrity of these organisms is maintained, not only by a tough, flexible surface pellicle, but also by a peripheral gelated layer of cytoplasm, often referred to as "ectoplasm." In the previous pressure studies on Paramecium, the organisms were not observed clearly during the compression period so that compression effects could not be distinguished from the effects of decompression; and in the previous studies on Blepharisma (Hirshfield *et al.*, 1957) no extensive variation in temperature was employed.

Materials and Methods

The ciliates, *Blepharisma undulans* and *Paramecium caudatum*, provided excellent material for this study because of their elongate shape which tends to become spheroidal when structural instability develops.

The original Blepharisma culture was obtained from Dr. H. I. Hirshfield; and the paramecia were derived from a mass culture maintained for many years at New York University. Both species were cultured in a lettuce-*Pseudomonas ovalis*

medium according to the method of Hirshfield (personal communication). The Blepharisma were maintained in an incubator at a temperature of 20° C., whereas the paramecia were kept at room temperature (25°–27° C.).

Periodically, single organisms were isolated and placed in the separate spots of a 12-spot Klein agglutination slide for five days. The contents of each depression were then transferred to a test-tube of lettuce medium which had been inoculated three days previously with *Pseudomonas ovalis*. Such Blepharisma clones were used for experimental purposes only from the seventh to the eleventh day after isolation. These gave fairly consistent data, but organisms from older cultures displayed a marked increase in pressure sensitivity. Paramecium cultures were more stable and gave fairly consistent results over a three-week period.

The constant-temperature housing, used with the pressure apparatus, has been described by Marsland (1950). The apparatus provides for a rapid build-up and release of pressure, and for constant microscopic observation while the organisms are under pressure. In the pressure bomb the protozoa were kept in view by confining them within a small plastic chamber (6.5 mm. diameter and 2 mm. depth) which was closed above and below by glass coverslips held in position by Lubriseal films.

Ten to thirty ciliates were placed in the chamber for each experiment, and the remaining volume (approximately 85 ml.) of the pressure chamber was filled with Brandwein solution. The duration of exposure to any given pressure was fifteen minutes. The pressures ranged from 7000 to 11,000 psi. and the temperatures employed were 12°, 15°, 20°, and 25° C. The organisms were counted several times during the progress of each experiment, and then at the end of the 15-minute period the percentage of cytolyzed individuals was determined. At least 60 Blepharisma in a total of four or more experiments were used at each pressure and temperature, at least in all critical ranges.

Results

Blepharisma: pressure-temperature effects on form-stability

Blepharisma from old mixed cultures were very sensitive to pressure. In such cultures (at 20° C.) 4000 psi. (lbs./in.^{2}) usually was sufficient to cause a rounding up of all the specimens and subsequently cytolysis occurred in over 75 per cent. Also, aging cloned cultures showed a steady decrease in resistance to pressure. In fact, during the fourth or fifth week after cloning, pressures of about 4000 psi. became sufficient to cause breakdown of the organisms, as was the case with the mixed cultures. Occasionally, young cloned cultures were found which displayed a similar super-sensitivity to the pressure-temperature conditions. Perhaps such clones were derived from a weak or aberrant individual. In any event they were not used for further experimentation.

Blepharisma from typical young cloned cultures showed little or no tendency to become rounded, regardless of the experimental temperature, until the pressure exceeded 7000 psi. Moreover, there was virtually no cytolysis within the 15-minute experimental period. At higher pressures, however, a number of the specimens first became rounded and then cytolyzed. Furthermore, the temperature of the

TABLE I

Percentage of cytolysis in Blepharisma after 15-minute exposure to various pressure-temperature conditions. These are the results of the individual experiments. In each experiment the number of lysed specimens is given in relation to the total number treated

T° C.	psi.			
	8000	9000	10,000	11,000
12	10–20 13–25 8–15 31–60 = 52%	12–15 15–20 27–35 = 77%	17–18 = 100%	—
15	4–20 3–12 9–23 7–15 23–70 = 33%	6–10 7–13 10–20 10–15 8–13 11–17 52–88 = 59%	9–10 23–25 11–12 13–15 20–22 76–84 = 91%	15–15 = 100%
20	5–25 3–15 2–10 3–10 4–13 2–10 3–10 22–93 = 24%	7–15 5–15 6–18 8–20 5–15 4–11 4–11 39–105 = 37%	6–10 9–10 14–15 9–10 13–15 51–60 = 85%	14–15 8–8 15–15 37–38 = 100%
25	3–20 2–26 2–16 2–22 9–82 = 11%	3–10 4–11 4–14 4–10 3–10 6–15 24–70 = 34%	5–6 7–8 17–20 10–12 13–17 9–12 13–16 74–91 = 81%	15–15 = 100%

experiment had a distinct influence upon the percentage of suseptibility, as is shown in Table I.

The character of the rounding and of the subsequent cytolysis varied somewhat in relation to the intensity of the pressure treatment and to the experimental temperature. However, under *critical conditions*—which may be defined as any pressure-temperature combination which yields just 50 per cent cytolysis in 15 minutes—the reactions were generally similar. Thus it is possible to describe the variations which occurred under sub-critical, critical, and super-critical conditions which, respectively, yielded more and more cytolysis within the experimental time.

The rounding and cytolysis reactions under slightly sub-critical conditions (9000 psi./25° C.) are shown in Figure 1. Under such conditions, generally speaking, the shortening seldom exceeded 25 per cent of the original length; the number of rounded specimens increased only gradually during the experimental period; and the tapered anterior end of the organism tended to retain a fairly close semblance of its original architecture. Generally, motility was absent or at least drastically retarded in the rounded specimens.

Lysis, as was the case under all conditions studied, occurred only subsequent to the rounding reaction. Under sub-critical conditions the time of the lysis was distributed quite evenly throughout the test period. In each specimen, however, the lysis was sudden, sometimes being initiated in the tapered anterior end (Fig. 1, C), and sometimes in the swollen posterior half, near the contractile vacuole. It appeared to involve a sudden rupturing of the cell surface and a disruption of the cytoplasm into a number of rounded free-floating pieces (Fig. 1, D). Occasionally, some of these protoplasmic fragments become motile after pressure was released.

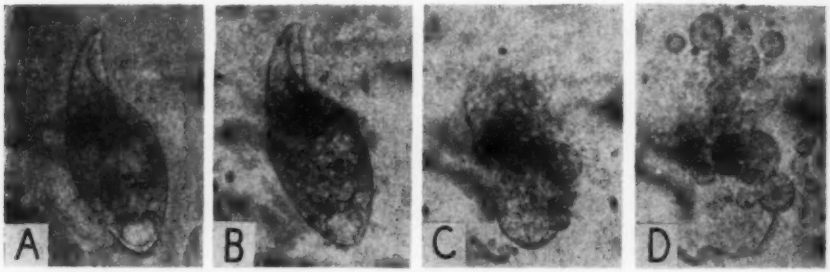

FIGURE 1. Blepharisma: rounding and cytolysis reactions under sub-critical conditions (9000 psi./25° C.). A and B: Gradual shortening of specimen; successive exposures taken 4 and 7 minutes after pressure build-up. C: Sudden cytolysis, exposure 30 seconds after B. D: Rounding up of cytoplasmic remnants, one minute after cytolysis. After decompression some of these fragments may become motile. Photographs retouched.

Under distinctly super-critical conditions, rounding and lysis developed rapidly and most of the susceptible specimens had reacted within the first five minutes. The shortening of specimens was distinctly greater, although often there was some persistence of the tapered anterior end up to the moment of lysis. The lysis was more complete; the protoplasmic fragments were smaller; these fragments showed less tendency to round up, and they did not develop motility subsequent to decompression.

Under intermediate conditions, in and around the critical range, the rounding and cytolysis reactions were intermediate in character.

Blepharisma: decompression effects

The sudden release of pressure, under critical or nearly critical conditions, gave rise, within two minutes, to an abrupt, further shortening of all the non-cytolyzed specimens, accompanied by a momentary stoppage of any persisting ciliary activity.

This shortening (Fig. 2) was more abrupt than the pressure-induced rounding. Immediately after shortening, a few specimens displayed sudden lysis, but this *decompression lysis* did not involve more than five per cent of the animals. In fact, most of the specimens regained their motility within some ten minutes, and after 30 to 200 minutes they presented a fairly normal form and appearance.

Blepharisma: pressure-temperature parameters of cytolysis

As may be seen in Table I, the percentage of cytolysis obtained at any given pressure represents a temperature-dependent value. The sensitivity to pressure cytolysis increases very definitely with decreasing temperature within the experimental range (25°–12° C.). This is shown more clearly when the data are plotted, as in Figure 3. Conversely, the resistance to pressure cytolysis increases with increasing temperature, as is shown in Figure 4. There it may be seen that the

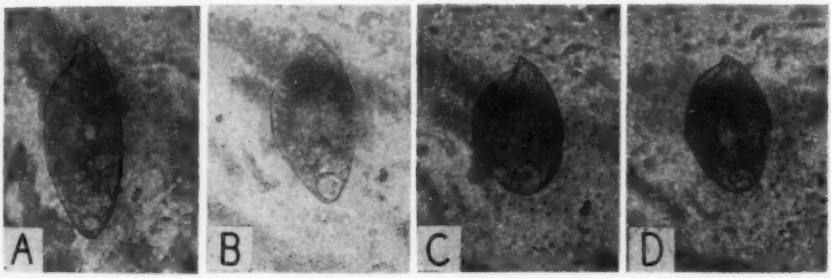

FIGURE 2. Blepharisma: shortening under pressure (slightly sub-critical conditions, *i.e.*, 9000 psi./25° C.) followed by rapid shortening after decompression. A: Exposure 13 minutes after pressure build-up. B: One minute after decompression. C: Two minutes later. D: Another two minutes later. Photographs retouched.

pressure which is just adequate to induce cytolysis in 50 per cent of the treated specimens increases regularly as the temperature increases, within the given range.

Blepharisma: pressure-centrifuge experiments

Quantitative measurements of the solational effects of pressure are difficult to obtain with Blepharisma. The pressure-centrifuge method, which has been used widely for other cells (Marsland, 1956), is not very suitable. The orientation of the specimens in the centrifugal field shows considerable variation, and the instability of form and cellular integrity at higher pressures gives further difficulty.

It was possible, however, to obtain qualitative data which showed unequivocally that pressure does induce solational changes in the cytoplasm of Blepharisma. Many of the specimens centrifuged for one minute at 5000 × gravity at 3000 psi. showed a distinct clearing of the centripetal half of the cell—by virtue of the centrifugal displacement of food vacuoles and other granular bodies—to a degree that was never found in control specimens, centrifuged simultaneously at atmospheric pressure.

Paramecium: comparative observations

Generally speaking, the pressure-temperature effects on Paramecium and Blepharisma were similar. However, there were two important differences: 1) Paramecium was distinctly more sensitive to pressure lysis, and 2) *decompression lysis,* which was almost negligible in Blepharisma, became very significant in Paramecium.

For Paramecium, the critical pressure for 50 per cent lysis was 2000–3000 psi. lower than for Blepharisma, at each of the two temperatures (20° and 25° C.) which were studied. Under such critical conditions (*e.g.,* 7000 psi./20° C.) the animals shortened moderately and displayed gradually diminishing, distinctly irregular locomotion, which ceased only if and when cytolysis occurred. Most of the cytolysis occurred during the last 5 minutes of the 15-minute compression period. Moreover, two somewhat different types of lysis were observed with roughly equal frequency. One type seemed to involve a detachment of the pellicle, with the formation of one or more large hyaline blisters which later broke, liberating the deeper granular cytoplasm (Fig. 5). The other type, in contrast, seemed to

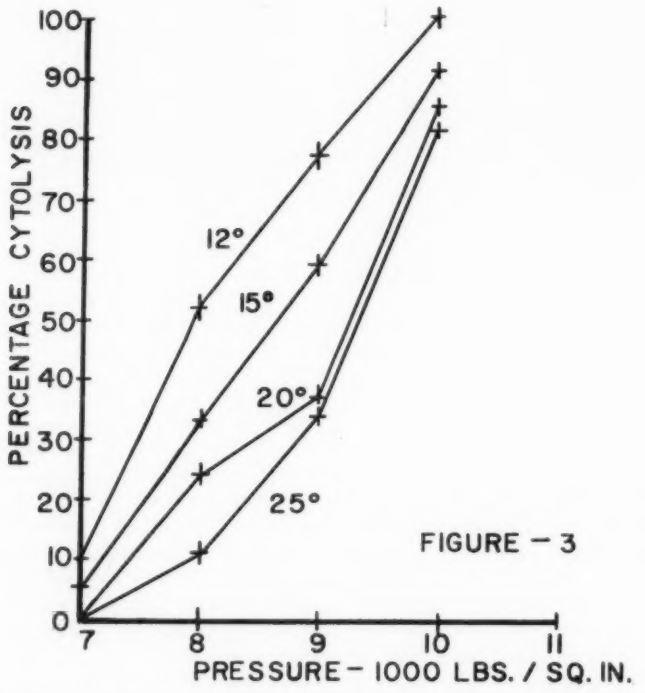

FIGURE 3. Blepharisma: percentage of cytolysis as a function of pressure, at four temperatures. Cytolyzed cells were counted exactly 15 minutes subsequent to pressure build-up.

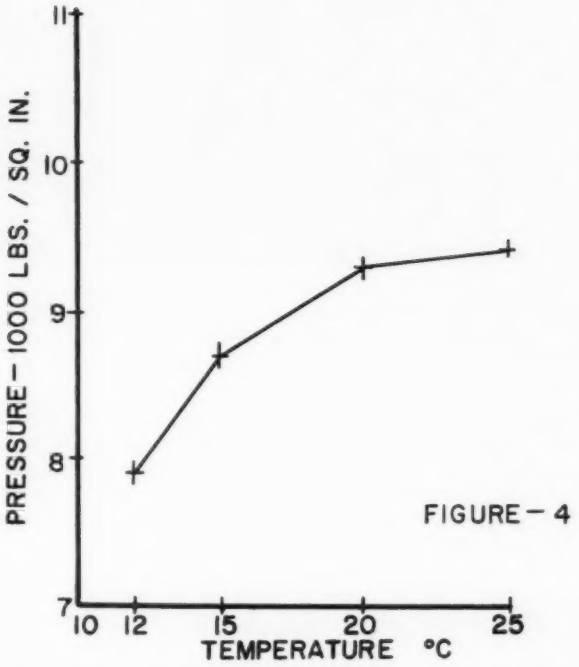

FIGURE 4. Blepharisma: critical pressure yielding 50 per cent cytolysis after 15-minute exposure, plotted as a function of temperature. The data of this figure are derived from Figure 3.

represent a more generalized breakdown of the cell surface in either the anterior or posterior half of the animal, with a less abrupt scattering of the granular cytoplasm (Fig. 6). With Paramecium, moreover, regardless of the conditions or type of cytolysis, there was very little tendency for the cytoplasmic remnants to round up, or to wall themselves off from the surrounding medium.

The decompression lysis under critical conditions usually involved more than half of the surviving specimens, particularly when the decompression was rapid (within one second). Sudden decompression was followed within about two minutes by an abrupt further shortening of all surviving specimens, followed immediately by a generalized cytolysis of the majority. Specimens that escaped cytolysis, on the other hand, gradually regained normal form and motility within 2–3 hours.

Under super-critical compression (8000 psi./20° C.), the degree of rounding was greater; and most of the lysis, which involved more than 60 per cent of the specimens, occurred during the first ten minutes of the compression period. Then, following rapid decompression, *all* surviving specimens shortened still more and quickly underwent cytolysis.

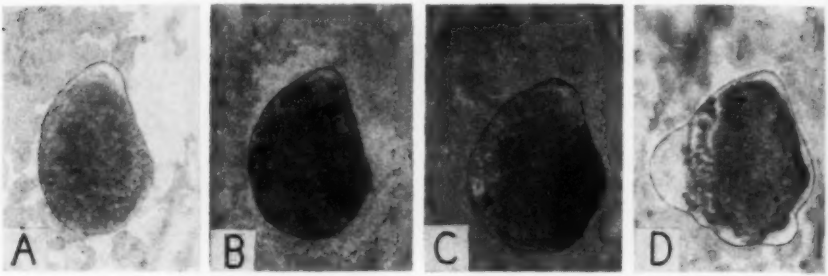

FIGURE 5. Paramecium: one type of pressure cytolysis (8000 psi./25° C.). A and B: Shortened specimen, photographed 9 and 10 minutes after compression. C: Two minutes later, cytolysis starting. D: 30 seconds later, showing hyaline blisters which are about to break. Photographs retouched.

Distinctly sub-critical conditions (5000 psi./20° C.) produced no clearly defined effects; but at 6000 psi. a slight degree of shortening was noted, although locomotion appeared to continue in normal manner. Abrupt decompression, under these conditions, produced a further sudden shortening of the specimens, but there was no cytolysis and usually the animals regained their normal form within one hour.

DISCUSSION

The problem of how pressure exerts its effects upon cellular systems has been approached from several angles. Regnard (1891) interpreted his results in terms of an imbibition of water by the ciliated cells. However, since no volume increase can be found in pressurized cells, this hypothesis has not been pursued. Hodapp and Luyet (1947) suggested a disturbance of the permeability mechanism and an injury of the neuromotor apparatus as the main factors involved in pressure lysis. However, as stated previously, they were unable to observe the organisms during the pressure period. As to their findings that temperature and the rate of application and release of pressure had no effect on lethality, at the high pressures they employed, it is probable that the decompression effects were very drastic and negated these variables.

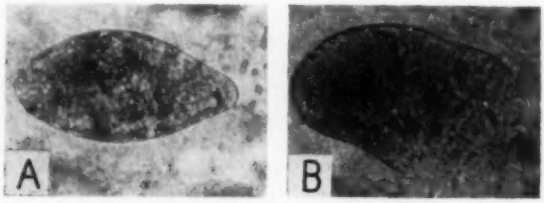

FIGURE 6. Paramecium: another more generalized type of pressure lysis (9000 psi./25° C.). A: Shortened intact specimen 5 minutes after pressure build-up. B: 5 minutes later, sudden cytolysis initially involving all of the anterior half of the specimen. Photographs retouched.

Pressure-temperature effects on cell form

A more fruitful approach, perhaps, is to interpret the observed effects on the cell form and integrity of Blepharisma and Paramecium in terms of the now well established action of pressure and temperature upon intracellular gel structures. To some extent this approach has been adopted by Ebbecke (1936), who had, however, very little experimental evidence. Moreover, Ebbecke postulated that pressure exerts its effect upon the gel system indirectly via an action upon cell metabolism, rather than directly *and* indirectly, as proposed by Marsland and Brown (1942).

It now seems reasonable to assume that the cortical cytoplasm of ciliates, immediately subjacent to the pellicle, is firmly gelated and that this plasmagel layer plays a significant role in helping to maintain the unique form of the particular species. Also, it seems possible that the plasmagel layer of the ciliate may possess contractile properties which can be instrumental in producing changes of form and orientation during normal locomotion.

Experimental evidence in regard to the foregoing question is not very extensive, however. A plasmagel structure is indicated, to be sure, by the fact that the peripheral layer of cytoplasm, in which the trichocysts are lodged, does not become involved in the protoplasmic streaming when cyclosis occurs. Also, it is frequently observed, not only in the pressure-temperature experiments, but also when Paramecium and other ciliates are exposed to toxic substances or merely flattened under a coverslip, that the pellicle may peel away from the subjacent cytoplasm and form a hyaline blister of large or smaller size. When this happens the granular cytoplasm, from which the pellicle has become detached, may persist, retaining its stability for a minute or two. Then it disintegrates and pours forth its granular components into the hyaline fluid which fills the blister.

All the evidence of the present experiments indicates that the shortening and rounding of the cells induced by suitably high pressure and modified by temperature are mediated by a solation of the plasmagel layer. Qualitatively, the susceptibility of this gel to pressure solation is established by the pressure-centrifuge experiments and quantitatively the pressure-temperature parameters of this gel system are very similar to those which have been established in various other protoplasmic gels (Marsland, 1956). Apparently a shortening and rounding of these elongate ciliated cells occur, under the agency of tensional forces in the cell surface, whenever the subjacent plasmagel structure is weakened below a certain critical resistance level.

Pressure-temperature lysis

Pressure lysis, apparently, is always preceded by a rounding of the cells; and, generally speaking, the more drastic the rounding the greater is the lysis tendency. It seems likely, therefore, that cell form and cell integrity may be determined by similar underlying factors.

A firmly maintained plasmagel structure would serve, most probably, not only to stabilize the total form of the cell, but also to preserve the orientation and spatial configuration of many of the microscopic and submicroscopic constituents of cell structure. Moreover, if the solation is drastic enough to allow for a rounding of the cell, the rounding itself tends to disturb and disorient the configuration of the

protoplasmic constituents. Cytolysis, perhaps, may involve a detachment of the pellicle from the subjacent plasmagel, with a concomitant disarrangement of the ciliary origins and trichocysts, or it may involve some other type of disorientation. In any event, it seems to occur whenever drastic solation occurs. Thus it is not surprising to note that preliminary dosages of UV-irradiation, utilizing wave-lengths which have a primary effect upon the proteins of the peripheral cytoplasm, predispose Blepharisma to pressure cytolysis, presumably as a result of a weakening effect upon the plasmagel structure (Hirshfield *et al.*, 1957).

Decompression lysis

This phenomenon, which was particularly conspicuous in Paramecium, can be interpreted, perhaps, in terms of a rapid post-pressure reconstruction and contraction of the plasmagel system. A similar phenomenon, in fact, has been described for Amoeba by Landau, Zimmerman and Marsland (1954). Many studies have shown that pressure solation is rapidly reversible upon decompression and the Amoeba study indicates that the newly reconstituted gel system tends to contract sharply, presumably as a result of an accumulation of metabolites which are not fully utilized during the pressure period (Landau *et al.*, 1954). In any event, both Blepharisma and Paramecium always showed an abrupt contraction about two minutes after release from any extensive critical or super-critical pressure-temperature treatment and, particularly in the case of Paramecium, this abrupt contraction was very frequently accompanied by cytolysis. Precisely why cytolysis should occur under these circumstances is problematical. It may be supposed, however, that such a contraction would tend to disrupt the surface architecture of the cell, especially if it occurs before a proper stabilization of the cell structure has occurred. Furthermore, these observations indicate that the peripheral gelated cytoplasm of the ciliate displays a potential contractility, and that this layer may play a role in effecting changes of form and orientation, during locomotion, and in performing the work of cell division.

Metabolic relationships

The increasing susceptibility of older cloned cultures in regard to pressure-temperature cytolysis raises some interesting questions. A continued source of metabolic energy appears to be necessary for the maintenance of protoplasmic gel structures (see Marsland, 1956); but why should such structures tend to be weaker in aging clones? Lettré (1952) has suggested that cell form and stability may be dependent upon the level of ATP reserve in the cell, but why should this tend to diminish with age? ATP-sensitive proteins, capable of forming potentially contractile gel systems, seem to be present in various relatively unspecialized cells—in the slime mold (Loewy, 1952 and Ts'o *et al.*, 1956), in sea urchin eggs (Mirsky, 1936), in fibroblasts and other tissue cells (Weber, 1955 and Hoffman-Berling, 1954), and in Amoeba (Landau *et al.*, 1954). At present, however, it is entirely problematical as to whether age-changes in the gel structure result from changes in metabolism, changes in the constituent proteins or, at least partly, from other unknown changes.

Summary

1. Two ciliates, *Blepharisma undulans* and *Paramecium caudatum*, were studied with reference to form stability and integrity (resistance to cytolysis) under varying conditions of hydrostatic pressure (up to 10,000 lbs./in.^{2}) and of temperature (12°–25° C.).

2. At lower pressures the specimens retained their elongate form, but at higher levels, depending on temperature, species, and age of the cloned cultures, the cells gradually become shorter and more rounded. Following this form change, cytolysis occurred in a varying percentage of the specimens. Older cloned cultures showed a greater and more variable susceptibility to the pressure-temperature effects, so that selected younger cultures were used for the quantitative evaluations.

3. For Blepharisma, the critical pressure, which gave 50 per cent cytolysis within a 15-minute compression period, displayed a distinct temperature dependence, being 8000 psi. at 12° C., 8700 at 15° C., 9200 at 20° C., and 9300 at 25° C. Paramecium, in contrast, showed a distinctly greater sensitivity, the critical pressure for 50 per cent cytolysis at 20° C. being some 2000 psi. lower than for Blepharisma.

4. Rapid decompression, following any critical or super-critical pressure treatment, produced an abrupt further shortening (contraction) of the specimens, accompanied by a cytolysis of some of the previously resistant individuals. For Blepharisma, decompression cytolysis involved only about 5 per cent of the animals. Paramecium, however, was much more sensitive and virtually 100 per cent became involved.

5. An interpretation of these changes in cell form and integrity is given in terms of pressure-temperature effects upon protoplasmic gel structure, particularly with reference to the solation of the peripheral plasmagel layer of the cytoplasm.

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OBSERVATIONS ON THE SYMBIOSIS OF THE SEA ANEMONE STOICHACTIS AND THE POMACENTRID FISH, AMPHIPRION PERCULA [1]

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The partnership between certain tropical damselfishes and sea anemones has excited the interest of students of natural history for almost a century. The most significant investigations of the symbiosis have been those of Sluiter (1888), Verwey (1930) and Gohar (1948), who have given us some knowledge of the ecology and behavioral characteristics of the animals. In 1947 Gudger reviewed all the observations that had been made up to that time, and in 1950 Baerends first speculated about the possible role of releasers in the maintenance of the association.

However, this symbiosis, like many others, still poses many unanswered questions. The physiological and behavioral mechanisms which maintain the animals in partnership have not been investigated with present-day techniques.

It has not been clear whether the fish responds to chemical, tactile or visual stimuli from the host, nor whether the behavior of the anemone is affected by stimuli from the fish. The mechanism whereby the fish is protected from the nematocysts of the host has been a mystery. In spite of the fact that it is generally supposed that nematocysts are not under nervous control but that they fire off independently upon adequate stimulation, several investigators have speculated that in such partnerships the presence of the fish in some way causes the coelenterate host to put its nematocysts "out of action" (Baerends, 1957, p. 262). The question remains whether the fish simply fails to provide adequate stimuli to discharge the nematocysts, or whether a factor is produced by the fish which markedly raises the threshold of discharge of the nematocysts and thus affords protection. Finally, it remains to be determined whether or not the fish is immune to the poison of the nematocysts.

Recently at Marineland of the Pacific it became possible to investigate the partnership between *Amphiprion percula* (Lacépède) and the giant anemone *Stoichactis* (Fig. 1). We directed our attention primarily to the physiological and behavioral mechanisms involved in the protection of the fish against the nematocysts of its host and in the course of the work were able to re-examine and re-establish some of the observations of Verwey and Gohar.

[1] Contribution No. 6, Marineland of the Pacific Biological Laboratory. This work was carried out under the contract of the senior author with the Office of Naval Research. We wish to express our appreciation to Marineland of the Pacific for its hospitality and facilities and to Dr. Cadet Hand for a tentative identification of our anemone.

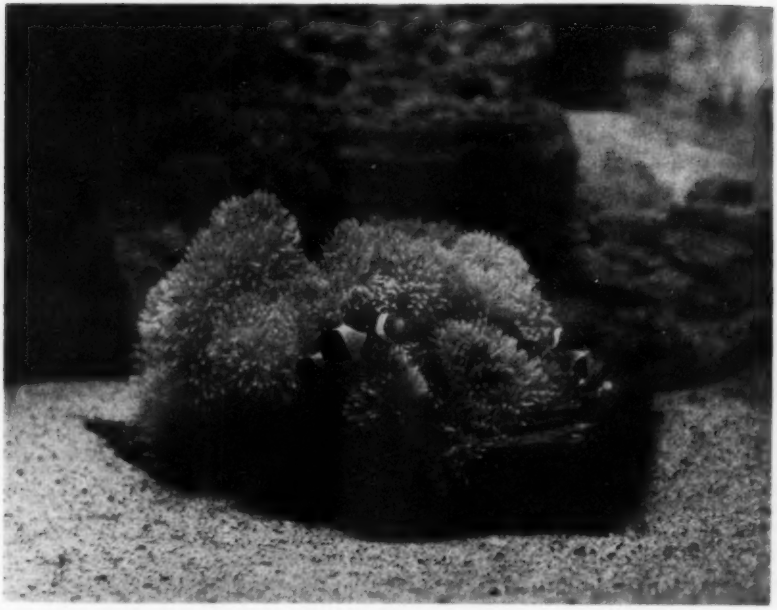

FIGURE 1. The anemone Stoichactis and two partner *Amphiprion percula*. Photographed in the exhibition aquarium at Marineland of the Pacific. Approximately × 1/6.

MATERIAL AND METHODS

Experimental fish were obtained on the reefs near Nasugbu, Batangas Province, Luzon, in the Philippine Islands by commercial collectors. Our single specimen of the host anemone was taken at the same locality. Verwey (1930) describes an anemone, probably identical with ours, from Batavia Bay, Java (Anemone 1, Plate XV, Fig. 2) which he says is colonized in nature by *Amphiprion percula* alone.[2] Ours was provided by the collectors specifically as the host of *A. percula*. At this writing it is still alive at Marineland. We believe it to be *Stoichactis kenti* (Haddon and Shackleton) although precise identification will not be possible until examination of the internal anatomy can be made after the animal is preserved.

The anemone was received at Marineland in January of 1957, and our experiments were started on September 3, 1957. Thus the animal was acclimated to Marineland sea water for a period of somewhat over 8 months. During the period of our observations it was maintained at 25° C. in a 60-gallon "photographic" redwood aquarium, which was so constructed that a sheet of glass could be inserted to isolate fishes from the anemone when desired.

We received a total of thirteen specimens of *Amphiprion percula*. The previous

[2] In the aquarium at Batavia this anemone was readily occupied by *Amphiprion akallopisus* and *A. polynemus*, in addition to *A. percula*.

history of these fish is totally unknown to us. Probably some or all of the animals were collected from anemones. However, according to Dr. José Montilla of the Division of Marine Fisheries in Manila, this species of Amphiprion does not always live in association with anemones in the Philippines; hence some of our experimental fish may have been free-living. Also, it is known that *A. percula* lives in association with at least two species of anemones (Verwey, 1930). Therefore the host habit of any that may have been commensal is also unknown to us.

Prior to the experiments, two fish, A and B, were kept in partnership with the anemone for several weeks. These gave us controls which we knew were "acclimated" to the anemone. Nine other fish (C to K) had been isolated from any possible sensory contact with an anemone host for a period of not less than six weeks. In the following experiments these are spoken of as "unacclimated" fish.

Two other *A. percula* (L and M) which had occasionally been put in with the anemone for exhibition purposes prior to our experiments were also used. One of these (L) was the largest animal in our sample, measuring 65 mm. standard length. Fish M, a small animal, was sacrificed in a physiological experiment.

The age and sex of our *Amphiprion percula* were not determined.

A single adult specimen (56 mm. standard length) of *Amphiprion frenatus* Brevoort was available for specificity studies. This fish had lived in the exhibition aquarium with the anemone for the eight months prior to our experiments but was never observed to enter it.

The most careful precautions were taken to maintain all glassware, forceps, scissors, dip nets, and other tools free from contamination with organic materials, because of the well-known sensitivity of nematocysts to such substances. All items employed in the manipulation of fishes or isolated anemone tentacles were scrubbed with detergent, washed in distilled water, dipped in ether-alcohol and allowed to dry without contact. Fish to be sacrificed were dissected with clean instruments in clean Petri dishes. Whenever possible, experimental fish were not handled at all but were trapped in the aquaria with clean 500-cc. beakers. When it was necessary to use a nylon dip net, the net was first boiled and rinsed.

Experiments on the discharge of nematocysts from isolated tentacles were conducted in clean watch glasses. A new tentacle was prepared for each test. Tentacles were isolated by clipping them off at the base with clean, fine-tipped scissors. They were stimulated mechanically with a clean glass rod drawn to a fine point, and electrically stimulated with a platinum wire-glass electrode drawn to a fine capillary point. The electrode was connected in a circuit with a standard inductorium, key, and a 6-volt dry cell. A small piece of aluminum foil dipped into the sea water in the watch glass served as the other electrode. Between each experiment, the watch glass, the glass rods, the platinum wire, and the capillary tube were washed with ether-alcohol; the capillary tube was refilled with clean sea water, and the aluminum electrode was replaced.

In certain experiments, one-cm. cubes of plastic sponge were used. These were cut from the center of a new commercial sponge by use of a clean single-edge razor blade.

Observations on the "Process of Acclimation"

It has been observed (Gohar, 1948) that the acclimation of an Amphiprion to an anemone may take a considerable length of time. The details of this acclima-

tion remain virtually unknown. We felt that careful observation of this process might give us insight into the mechanism which protects the fish from nematocyst discharge.

Accordingly, a series of nine experiments were performed in which we introduced individual unacclimated *Amphiprion percula* into the observation tank with the anemone. These tests revealed a fairly stereotyped series of events which terminated in the acclimation of each new fish to the anemone. The results of these experiments are summarized below.

An unacclimated fish introduced into the tank a foot or so away from the anemone, usually approached the anemone within a few minutes and began to swim under the disk, around the column, and occasionally over the top of the disk a centimeter or more away from the tentacles. Such fish spent most of their time under the disk at this stage and sometimes were seen nibbling at the column of the anemone. Most fish seemed to "recognize" the anemone within a few minutes and swam toward it. However, in two tests, two fish failed to react noticeably to the anemone for 20 and 27 minutes, respectively. In both cases another fish was introduced directly onto the disk of the anemone where it shortly took up residence. In both tests the unreactive fish then came rather quickly toward the anemone, apparently in response to the fish already in occupancy, and began the characteristic acclimation process.

As the process proceeded, passage over the disk became more and more frequent and the "acclimating" fish moved closer and closer to the tentacles. Swimming was accomplished by a distinctive series of slow vertical undulations, in which the tail was usually held a little lower than the rest of the body. Eventually, on one of these trips over the disk, the fish would touch a tentacle or two, usually with the ventral edge of its anal fin or the lower margin of its caudal fin. Commonly this resulted in a moderate adherence of the tentacle to the fin and contraction of the tentacle. The fish then jerked itself free with a violent flexure of its body and usually raced off the disk. Not all newly introduced Amphiprion caused clinging upon their first contact with tentacles, but it was the general rule. However, this adherence failed to deter the fish, which nearly always returned immediately to the anemone, either under the disk or over the tentacles. In our experiments the time from initial introduction until the first physical contact between fish and anemone varied from less than 1 minute to 65 minutes.

After this initial contact the fish typically came closer and closer to the tentacles, touching them with increasing regularity. The reaction to the clinging of tentacles became less and less violent until a sudden flexure of the animal's body was the only reaction given by the fish. Mouthing or nipping of tentacles was often observed in this and later stages.

The clinging and contraction of tentacles upon contact with the fish gradually became less until it ceased altogether. At the same time the fish began to swim deeper among the tentacles, using the same slow undulating movements as when it had cruised above the disk.

Once the fish was swimming in fairly constant contact with the tentacles of the anemone, a very striking change in its behavior occurred. The general speed of swimming suddenly increased until the Amphiprion was dashing back and forth over the disk of the anemone, flailing unreactive tentacles aside with violent move-

ments of its body. Often the fish raced beneath the anemone and appeared in one of the folds of the disk margin, its head completely ringed in tentacles. The fish frequently maintained this vantage point for a few seconds, holding position with rapid alternate fanning movements of its pectoral fins, after which it might dash onto the disk again for another foray among the tentacles. The powerful swimming typical of this stage of the acclimation process was accomplished by rapid and strong lateral body flexures. The impression given by the swimming behavior of the fish after final acclimation was that the fish was "bathing" its entire skin surface among the tentacles.

At this point we considered the fish to be fully acclimated to the anemone, since no further clinging or tentacle contraction appeared. The time required for complete acclimation varied from about one minute to nearly three hours, with an average time of one hour.

If a fully acclimated fish was removed from the anemone and its fins or body carefully scraped with a scalpel, and then returned to the anemone, the scraped areas caused both clinging and tentacle contraction. However, fish treated in this manner did not then begin the acclimation process anew but stayed among the tentacles until clinging waned and disappeared. These fish gave evidence of discomfort from the clinging tentacles by jerking themselves free. They did not, however, rush off the disk. It would seem that treating the fish in this way partially broke down their protection.

Acclimation involves development of visual recognition of the anemone by the fish. This was demonstrated by removing fully acclimated fish from the anemone and placing them in a compartment of the observation tank separated from the anemone by a heavy glass sheet. Incoming water was introduced into the isolation compartment, flowed over and around the partition, and was discharged from the compartment containing the anemone to prevent chemical gradients from occurring which could guide the fish. In every case acclimated fish oriented strongly toward the anemone which they could see through the glass, by gathering at the glass nearest it and swimming up and down with their heads directed toward their host.

The behavior of an Amphiprion which has been resident for a time in an anemone is somewhat different from that of a newly acclimated animal. The general level of activity becomes lower though such a fish normally moves much more rapidly than an unacclimated fish. After acclimation of the fish is complete the anemone tends to become a strongly defended territory. Acclimated fishes often refuse to leave the anemone's folds even if it is lifted from the water.

Experiments on Protection Against the Host

In these experiments we wished to determine initially whether the presence of the fish close to but not in contact with the surface of the anemone had any *observable* effect on the anemone.

Experiment No. 1. A ¼-inch I.D. plastic tube was cleaned with alcohol-ether. A small *A. percula* was slipped into the tube and shaken down it until it protruded slightly from the end. When the fish was held as close as ½ mm. from the tentacles, they showed no reaction whatever. A similar test with a control *Fundulus parvipinnis* gave identical results. Contact of a single tentacle with the Fundulus resulted in immediate massive discharge and clinging.

No interaction at a distance between the partners or between prey and anemone could be observed.

Next, in the hope that we might be able to identify and localize the mechanism of protection, we designed the following experiments in which direct stimulation of the anemone was employed.

Experiment No. 2. As a control, we investigated the reaction of the anemone to stimulation with a clean, flame-polished glass rod. In a number of repeated tests we saw that such stimulation caused "clumping" of the tentacles, marked adherence to the rod (discharge of nematocysts), retraction of the tentacles, and retraction of the lobe of the disk in the vicinity of the point of stimulation. Far greater mechanical stimulation and agitation of tentacles and disk by Amphiprion produce no noticeable response from the anemone.

Experiment No. 3. We trapped an Amphiprion in a beaker and held it by the lower jaw in the tips of a pair of fine-tipped forceps. Twice we drew it forcibly across the disk of the anemone, bringing it into violent contact with the tentacles. There was no discernible reaction from the anemone. The fish when released immediately entered the tentacles in a normal manner and "bathed" itself among them.

An adult *Fundulus parvipinnis* was brought into contact with the anemone and was seized in the characteristic way, involving widespread adhesion, tentacle contraction, and infolding of the disk.

An *Amphiprion percula*, trapped in a beaker and held with forceps by the jaw, was brought into contact with a large specimen of the eastern Pacific anemone, *Anthopleura xanthogrammica*. There was immediate widespread clinging so that the fish had to be pulled forcibly from the anemone.

Experiment No. 4. An Amphiprion was sacrificed, and we cut a cross-sectional piece of flesh, including skin, from it with a carefully cleaned scalpel. We made a similar preparation from Fundulus. The two preparations were placed next to each other on the disk of the anemone. The flesh from Amphiprion was slowly worked to the edge of the disk and cast off, while the Fundulus meat was enveloped and ingested. The experiment was later repeated with similar results.

Experiment No. 5. We caught an Amphiprion, placed it in a clean Petri dish and killed it by severing the head. We then dissected off a strip of skin, taking the greatest care to prevent contact of both surfaces of the strip with other skin surfaces. We brought this piece of freshly-removed skin into contact, *on its outer surface*, with several tentacles of the anemone. No clinging occurred except for slight adherence at the edge of the piece of skin. When the skin was brought into contact *on its inner surface*, the tentacles immediately clung strongly to it.

This experiment was repeated twice with identical results. Strips of skin from the same fish were used.

Experiment No. 6. We heated the two pieces of skin used in the preceding experiment to 90° C. for ten minutes in sea water in separate clean test tubes. The preparations were cooled. When we brought the outside surface of these heat-treated pieces into contact with tentacles, clinging immediately occurred.

Experiment No. 7. A ½-cm. cube of muscle without skin was cut from the caudal peduncle of the *Amphiprion percula*, taking great care not to bring it in contact with skin surface. It was placed on the disk of the Stoichactis, and was

immediately seized. The tentacles clumped around the piece and infolding of the disk margin occurred. The anemone's response differed in no discernible way from its response to Fundulus meat.

Experiment No. 8. Four cubes were cut from a commercial plastic sponge.

In the following tests, the "clinging reaction" of a small group of tentacles was tested. When the plastic cube was brought in contact with the tentacles, the reaction was classified arbitrarily from 0 (no clinging) to ++++ (very strong adhesion). In each test a different group of tentacles was selected. The time required for release of the cube was noted.

a. A clean control cube: tentacles retracted; clinging 0-+; time of release < 1 second. This control was repeated several times with identical results.
b. A similar cube of which all surfaces had been rubbed over the skin of *Amphiprion percula:* results identical with the control. This test was repeated several times with similar results.
c. A cube rubbed over the skin of *Amphiprion frenatus:* clinging ++; retraction of tentacles, release time 20 seconds.
d. A cube rubbed over the skin of an adult Garibaldi, *Hypsypops rubicunda* (an eastern Pacific *pomacentrid* fish): clinging ++++, released after 2 minutes 45 seconds.
e. A cube rubbed over the skin of *Fundulus parvipinnis:* clinging ++++, released after 3 minutes 45 seconds.

Experiment No. 9. Four new cubes were cut. Two of these were rubbed over *Amphiprion percula.* One clean cube and one mucus-covered cube were heated to 100° C. for ten minutes in a dry oven and cooled.

a. The clean control cube: tentacles retracted; clinging 0-+, time of release < 1 second. Heavy pressure caused sufficient clinging to hold the cube for as long as 4 seconds.
b. A clean cube, heat treated: results identical with control.
c. A mucus-covered, unheated cube: tentacular retraction; clinging 0, even under strong pressure; time of release, immediate.
d. A mucus-covered, heat-treated cube: identical with control (a).

Identical results were obtained in a second series of tests. In this experiment we see that stronger mechanical stimulation than was used in Experiment 8 induced clinging of brief duration in a control sponge. If there was a coating of *Amphiprion percula* mucus on the sponge, clinging could not be induced even with strong pressure. But if the coating of Amphiprion mucus was heat-treated, its protective effect was obliterated.

The effect of heat was also shown in Experiment 6.

Experiment No. 10. On May 14, 1958, while the anemone was located in a display tank, two large groups of eggs were found attached to the rock occupied by the anemone. One patch was being guarded by an adult goby *Bathygobius soporator* (Cuvier and Valenciennes) and the other patch, which was attached in a crevice directly beneath the anemone, was guarded by two adult *Amphiprion percula,* which had been allowed to become resident in the anemone. The eggs of both species were tested for protection against the nematocysts of the anemone.

When the intact egg of the goby was touched against a tentacle, clinging occurred, and the tentacle bent into a clump with four or five other tentacles. No

movements of the disk were noted. When eggs were released in the water over the anemone and allowed to drift onto the tentacles the same effects were produced.

When these tests were repeated using Amphiprion eggs, the following results were obtained. Even when an egg was pressed against a tentacle with sufficient pressure to bend the tentacle no clinging resulted. Eggs dropped onto the disk through the water caused no reaction. Quite evidently Amphiprion eggs are as effectively protected as the adult.

Experiments on the Discharge of Nematocysts from Isolated Tentacles

Our next experiments were designed to determine whether Amphiprion mucus raised the threshold of nematocyst discharge. Isolated tentacles were stimulated mechanically or electrically while being observed through a dissecting microscope.

Mechanical stimulation. Pantin (1942) showed that direct mechanical stimulation of the isolated tentacle of *Anemonia sulcata* with a clean glass bead failed to cause discharge. Experiment No. 2 showed that stimulation of the *in situ* tentacle tip of Stoichactis with a smooth flame-polished glass rod results in clinging. Isolated tentacles of Stoichactis appear to be more sensitive to mechanical discharge than those of Anemonia. Even when the greatest care was taken in transferring a tentacle to a clean watch glass in clean sea water for isolation and stimulation, its tip very frequently stuck to the bottom of the glass for a few seconds.

In our preparations mechanical stimulation was effected by fine glass rods or by using the tip of the capillary tube of the glass electrode. Variation in the sensitivity of tentacles, the ease with which nematocysts could be mechanically discharged and our inability to deliver mechanical stimuli of precisely controlled intensity made it difficult to obtain a truly quantitative picture of threshold changes and intensity of discharge.

Our observations on the results of mechanical stimulation by the capillary tube of the electrode may be summarized as follows (discharge classified arbitrarily from 0 to ++++):

a. *Stimulation with the clean capillary tube:* An initial stimulation (light touching) at the tip of the tentacle typically produced a moderate discharge (++). Similar stimulation halfway between the tip of the tentacle and its cut base results in a lighter discharge (+). Repeated mechanical stimulation at both points results in progressively less discharge. Reduction of the discharge is not due to exhaustion of the nematocyst supply, as subsequent electrical stimulation produces massive discharge at the same points.

b. *Stimulation with the tip of the capillary tube covered with a pad of Fundulus mucus (control):* Initial light mechanical stimulation at the tip produced massive discharge (++++) and clinging to the mucus pad.

c. *Stimulation with the tip of the capillary tube covered with mucus from Amphiprion percula:* Light stimulation of both tip and middle of the tentacle produced no discharge (0). If the tentacle was held in place by a clean glass rod and stimulated at another point by the mucus-covered capillary tube so forcibly as to deform the tentacle, the discharge of a few isolated nematocysts occurred but *no clinging resulted.* The effect of the pad of Amphiprion mucus appeared to be

limited to the area in contact with the mucus, for if an uncovered portion of the glass tube came in contact with the tentacle, discharge would occur at this point but not at points protected by the mucus pad.

d. *Stimulation with the capillary tube covered with a pad of mucus from Amphiprion frenatus:* Light touch at the tentacle tip → ++. A touch at the side of the tentacle → 0. This test did not appear to be significantly different in results from control (a).

e. *Stimulation with the capillary tube covered with mucus from the base of Stoichactis:* Not significantly different from control (a).

Electrical stimulation. Stimulation by faradic current was produced according to the standard method described above. The single excised tentacle, in clean sea water, was first tested for mechanically-induced discharge by light contact with the electrode at a point halfway between its tip and base. The tentacle was then given a series of three-second bursts of faradic stimulation at the same point, starting with the inductorium at its lowest setting (12).

Table I shows the threshold and intensity of discharge under different conditions in a series of tentacle preparations. Intensity of discharge was arbitrarily classified from 0 to ++++. The sensitivity of the control series in which mucus was absent varied widely. It appears that this variation in sensitivity reflects variation in the threshold of the different preparations, since after the initial mechanically-induced discharge, stimulation by pressing the electrode against the side of the tentacle elicited no further discharge in the four preparations. When a pad of Fundulus mucus was placed over the tip of the electrode, the very lightest mechanical contact of the electrode tip with the side of the tentacle elicited some discharge (+). Hence, if the threshold of electrically-induced discharge had been lowered by the mucus, it could not be discerned. Intensity of discharge at high levels of electrical stimulation did not appear to differ from the controls. When pads of mucus from *Amphiprion percula, A. frenatus* and the anemone itself were used, results did not differ significantly from the controls.

It is quite clear that the presence of mucus from the partner fish did not raise the threshold of electrically-induced discharge of nematocysts. It is also interesting to note that maximum discharge in all cases was elicited within a narrow range of inductorium setting (4–5).

Discussion

The above investigations were principally directed toward understanding the physiological and behavioral mechanisms which maintain the animals in partnership and which protect the fish from the nematocysts of its host.

The reactions of unacclimated fish to the anemone were described in detail. These reactions differed considerably within our sample, but it must be remembered that the history of our nine fish was unknown to us. Some may have been free-living and some commensal with other species of anemone. However, the entire sample ultimately became acclimated to the Stoichactis. There is unquestionably a bond which attracts *A. percula* to this anemone and keeps the fish in it, once the acclimation process is completed. This process was first observed by Gohar (1948) who says (p. 39): "Fish of the commensal species may *develop* partnership with such anemones as *Discosomum giganteum* by cautiously approaching it. The

TABLE I

Threshold and intensity of discharge under faradic stimulation in isolated Stoichactis tentacles

Mucus pad....	Control (mucus absent)				Fundulus			*A. percula*			*A. frenatus*	*Stoichactis*
Test.....	1	2	3	4	5	6	7	8	9	10	11	12
Induct.					—	—	—	—	—	—	—	—
12	0	0	0	0	—	—	—	—	—	—	—	—
11	0	0	0	0								
10	0	0	0	0	(+)	(+)	(+)	0	0	0	0	0
9	0	0	0	0	(+)	0	(+)	0	0	0	0	0
8	0	+	0	+	(+)	0	++	0	?	+	0	0
7	0	+	0	++	+	+	++	0	+	+	+	0
6	+	++	+	+++	+++	++	++	+	++	+++	+++	+
5	+++	+++	++	++++	++++	+++	++	++	+++	++++	+++	+++
4	++++	—	++++	—	++++	+++	++++	++++	++++	—	++++	++++

association is completed in one to a few days." Once the association is completed the bond is stonger; acclimated fish rarely wander away from the anemones, while unacclimated ones may wander all over the aquarium. In the course of the acclimation process we observed the "cautiousness" noted by Gohar. A fish touches the tentacles, often sticks at first and flees. But it *keeps coming back*, making more and more contact until no tentacular clinging occurs. We have not as yet identified those signs which attract the fish, beyond confirming Verwey's observation (1930) that the fish respond to visual cues. Since our anemone was kept in still water, part of the visual cue to an unacclimated fish may have been absent, since in nature the Stoichactis must be in almost constant motion in its shallow water habitat. We have not as yet investigated the possibility that specific chemical releasers from the anemone may be an important part of the bond. Tactile stimuli may also be important, for the fish appears to "seek" contact with the anemone during the acclimation process. The process of acclimation may be recognized by the action of the fish of bringing more and more of its body in contact with the host. The strength and effectiveness of stimuli from the anemone certainly affect the rate of attainment of the ultimate equilibrium between the partners, which is the consummation of the acclimation process.

The behavior of the anemone in relation to the fish was also carefully observed. Some writers have claimed that the commensals, even without contact, affect the behavior of the tentacles. Crespigny (1869) said (p. 10): " . . a Premnas now passes over the anemone and immediately the tentacula become erect and diverge, while their extremities become clubby. . . ." Herre (1936), working with the symbionts used in our investigation, says (p. 167): "But when an *Amphiprion* darted in among the beautiful but dangerous tentacles, they curled away from the intrepid invader." We have never observed any such action at a distance, in spite of efforts to elicit some response by bringing an acclimated Amphiprion, held by its lower jaw or immobilized in a plastic tube, within a fraction of a millimeter of the host. In the former test, water currents from the fish's pectoral fins gently waved the anemone's tentacles, but no such response appeared as that described above. Even when an acclimated Amphiprion was dragged across the disk, no response occurred which was not attributable to mechanical disturbance; we suspect that the observations of Crespigny and Herre were merely the result of water currents.

Gohar implies that the fish in some way affect the nerve net of the anemone when he speaks of the activity of the fishes appearing (p. 38) "as if they were . . . sympathetically caressing" closed anemones so that they opened. This observation was also made by Verwey (1930). Gudger feels that such behavior involves a certain "gentle massage." This activity may be effective in bringing the anemone back to its expanded state, and if so, then the fish is affecting the neuromotor apparatus of the host. But the purposive implication is unwarranted, since the behavior of the fish is probably not very different from that when the anemone is already expanded and may be under the control of the same stimuli as those eliciting typical "acclimating activity."

We have produced other evidence that the presence of the fish may affect the neuromotor apparatus of the host, for contact by an unacclimated fish may result in localized retraction of tentacles, and, if stimulation is particularly strong, a slight infolding of the disk may occur. This reaction is similar to that elicited by contact

with prey, but is not as intense, involving briefer tentacular retraction and a weaker, more localized infolding of the disk. The frequently violent activity of the acclimated fish has no apparent effect on the anemone whatever. It is as though a physiological barrier had been set up during the acclimation process. We believe that the weak response of the anemone to an unacclimated fish indicates that the fish has not yet reached a state in which it fails to stimulate the host and that low intensity stimuli of the same nature as those received from prey are "getting through." It seems apparent that in the acclimation process, repeated contact with the anemone is necessary for the establishment of both the physiological protective barrier and the "bond of association" between the animals. We do not know the exact nature of the barrier. If one places a skinless piece of Amphiprion meat on the disk it is consumed, while a piece with skin attached is rejected and ultimately falls off the disk. Furthermore, if a piece of Amphiprion meat with skin and a piece of Fundulus meat with skin are placed side by side on the disk the former is rejected and the latter consumed. It appears as though the anemone "discriminated" between them. From this we are forced to conclude that a factor is present in the skin which affects the stimulus-response chain in the anemone. But the factor may do this indirectly by preventing nematocyst discharge, if, for instance, the normal feeding reactions depend upon the reception of information from receptors in the tentacles which are sensitive to bursts of nematocyst discharge, or to substances released from prey that has been "stung."

We are persuaded that the protection of the fish against its host's nematocysts does not involve a simple inability on the part of the fish to give adequate stimuli for discharge. We have shown that (1) the strongest stimulation of an isolated tentacle by a glass rod covered with Amphiprion mucus results in little or no discharge whereas like stimulation by a clean rod causes a burst of nematocysts; (2) when an Amphiprion is dragged across the disk of the host no discharge or adherence occurs; (3) the inner surface of a piece of Amphiprion skin sticks immediately to the tentacles, while the outer surface does not; (4) heat-treatment of the skin abolishes the protection; (5) a sponge covered with Amphiprion mucus will not stick to the tentacles, even when firmly pressed against them, while a clean sponge will; (6) heat-treatment of a mucus-covered sponge destroys the protection; (7) Amphiprion is immediately seized by another anemone (Anthopleura). All these observations argue for the existence of a heat-labile factor present on the outer surface of the skin of Amphiprion, which raises the threshold of discharge of nematocysts in the host Stoichactis.

What is the function of the behavioral process we call acclimation? We believe that this process, which other workers have suggested serves to change the condition of the anemone, is more probably a mechanism which changes the condition of the fish as the result of repeated contact between it and the anemone. It remains to be determined whether the fish has immunity to the nematocyst contents and whether acclimation has any relation to the maintenance of this immunity. There is a possibility, though we cannot offer conclusive proof, that acclimation may be related to changes in the mucus coat of the fish. Frequently, prior to complete acclimation, the protection of a fish is not perfect. Perhaps increasing contact with the anemone induces a greater general secretion of mucus or, specifically, more of the active principle in the mucus. Rough handling of the fish renders it susceptible

to localized stinging, which may result from "breaks"in the protective mucus coat. During the early stages of acclimation the fin tips are the sites of nearly all localized clinging. These edges are precisely the areas which are first brought carefully into contact with the anemone. Similar clinging occurs at the site of a wound in a damaged fish, but after a short time, if the wound is not great, clinging no longer occurs. This may indicate the spread of mucus over the wound, renewing the integrity of the protective coat. It would seem that the characteristic fluttering movements of Amphiprion when on the disk of the anemone would be particularly effective in spreading mucus over the various sharp fin edges.

Clearly there remain many unsolved problems. It appears that the protective principle in the mucus coat takes its effect locally, is fast-acting and specific. It would be of interest to determine its rate of decay and to find out whether, after cessation of contact between a tentacle and Amphiprion mucus, there is some effect, however brief, on the threshold of nematocyst discharge. One would wish to know a good deal more about the chemical nature of the principle and whether it is present in other fishes such as Nomeus, the commensal of the Portuguese Man-of-War, Physalia.

Summary

1. The behavioral process is described whereby the fish *Amphiprion percula*, after long isolation from the anemone Stoichactis, effects its association with the host.

2. This process appears to involve a gradual acclimation to the host, brought about by increasing contact with the host's tentacles. This appears to effect the establishment of both the "bond" and the physiological protective barrier between the animals.

3. Evidence is presented that an active principle is present in the mucus secreted on the outer surface of the integument of Amphiprion which raises the threshold of mechanically-induced discharge of the host's nematocysts. This factor does not affect the threshold of electrically-induced discharge. It is fast-acting, specific in its effect and heat-labile. It is not present in the muscle of the fish.

4. After contact between the host and an acclimated commensal no feeding reactions can be observed in the anemone such as occur when similar contact is made between Stoichactis and prey fish or between other anemones and Amphiprion. It is possible that this "inhibition" of the anemone may be the result of a direct effect on the nervous system by the active principle. However, it would seem more probable that this absence of feeding reactions even on violent contact may depend upon the fact that nematocysts are not discharged. Perhaps stimuli from receptors in the tentacles sensitive to nematocyst discharge or to substances from "stung" prey are necessary for the initiation of feeding reactions.

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A COMPARISON OF THE EFFECTS OF GOITROGENS ON THYROID ACTIVITY IN TRITURUS VIRIDESCENS AND DESMOGNATHUS FUSCUS[1]

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Salamanders of the genus *Triturus* have been used widely in studies of the histology, cytology, seasonal variation, and responses of the endocrine glands. Relatively few investigations of these matters have been made with members of other urodele genera. It would be interesting to know to what extent findings based on study of an exclusively aquatic newt are applicable to urodeles that are terrestrial in habit. There is some indication that the thyroid of *Triturus* may differ significantly in at least one respect from that of the terrestrial salamander, *Desmognathus*, namely, in the histological changes elicited by treatment with the goitrogenic drug, thiourea. Adams (1946) found that a high dosage and a long period of treatment are required to bring about hypertrophy and hyperplasia of the thyroid gland of *Triturus viridescens* whereas Fisher (1953) and Wheeler (1953) reported that the thyroid of *Desmognathus fuscus* responds much more readily and typically to this goitrogen. Since, however, the doses, environmental conditions, and length of treatment differed considerably in these studies, it is not possible to conclude with any assurance that *Triturus* is refractory to the effects of thiourea.

In our experiments a comparison is made of the responses of these two salamanders to treatment with two different goitrogens, thiourea and potassium perchlorate. The effects of these goitrogens on radioiodine uptake by the thyroid and on the histology of the gland were the basis for the comparison.

Materials and Methods

Specimens of *Triturus* (*Diemyctylus*) *viridescens viridescens* (Rafinesque) were collected on October 20, 1955, from a pond near Monterey, Virginia. Specimens of *Desmognathus fuscus fuscus* (Rafinesque) were taken from beneath stones at the edge of a small stream on a thickly wooded hillside near Oliver Springs, Tennessee, over the period June 23–July 23, 1956. All animals were maintained in the laboratory for a minimum of two weeks before being subjected to experimental treatment. Both before and during treatment they were fed every other day, the *Triturus* with ground lean beef fortified with cod liver oil and calcium phosphate, and the *Desmognathus* with live meal-worm larvae. Throughout the

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[2] University of Virginia.
[3] The Catholic University of America.
[4] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

experimental period the specimens were kept in a constant-temperature room at 19.0° ± 1.0° C.

Goitrogens were injected into the body cavity on alternate days. Some animals were given injections of 0.1 ml. of a 1.0% aqueous solution of thiourea; others received injections of 0.1 ml. of a 0.2% aqueous solution of potassium perchlorate. Controls were given injections of 0.1 ml. of distilled water. The injections were made with a 27-gauge needle introduced into the body cavity through the muscles at the base of the hind leg. We chose this site to prevent the loss of fluid that sometimes occurs when injections are made directly through the abdominal wall. Successive injections were always given on alternate sides.

The uptake and turnover of radioiodine in the thyroids of experimental and control animals were observed by the following method: The animal to be studied was injected intraperitoneally with 0.1 ml. of 10% Holtfreter's solution containing 50.0 μc/ml. of I^{131}. At fixed intervals over a 2½-day period thereafter the animal was anesthetized in an aqueous solution of tricaine methane sulfonate (1 part in 1000) and the radioactivity of the thyroid and heart regions was measured by a scintillation counter consisting of a 1.5-inch NaI crystal cemented to the window of an RCA type-5819 photomultiplier tube and a conventional amplifier and binary scaler. The crystal and photomultiplier were mounted in a lead cylinder 5.2 cm. thick with a collimating slit measuring 4.0 by 12.0 mm. The ventrum of the anesthetized animal was apposed to the lead cylinder with the region to be counted directly over the slit.

The procedure just described was evolved from a series of preliminary experiments carried out to ascertain the optimal dosage of I^{131} for reliable counts, the time required for maximum uptake by the thyroid, the extent of individual variation in uptake among control animals, and the possible effects of frequent anesthetization on the uptake. In one preliminary experiment, counts were made at eight successive levels on the anterior-posterior axes of I^{131}-injected animals from the snout to the base of the tail, giving a profile of radioactivity. Relatively high counts were obtained in the region of the thyroid and in the abdomen. The heart region was selected as representative of the tissues in general (other than the gut) for comparison with the thyroid region. The counting on these two regions was done by centering the thyroid or the heart, as located with relation to external anatomical characters, over the collimating slit. During the experiment the mean background count was 1.68 counts per second. It did not vary significantly over the 60-hour counting period.

At the conclusion of the measurements of radioactivity the animals were killed. The lower jaws, containing the thyroid glands, were fixed in a solution containing equal parts of Bouin's solution and ethylene glycol monethyl ether. Sectioned thyroids were stained with Harris' hematoxylin and Ponceau de zylidine-orange II (Gray, 1952) for histological study.

The results to be described here were based on information obtained from the study of 72 salamanders divided into two series, one made up of animals that received injections for 30 days and one of animals that had 46 days of treatment. Each series consisted of six groups of six animals each; one group of control, one of thiourea-treated, and one of perchlorate-treated specimens for each of the two species.

Results

1. *Effects of thiourea and perchlorate treatment upon the histology of the thyroid*

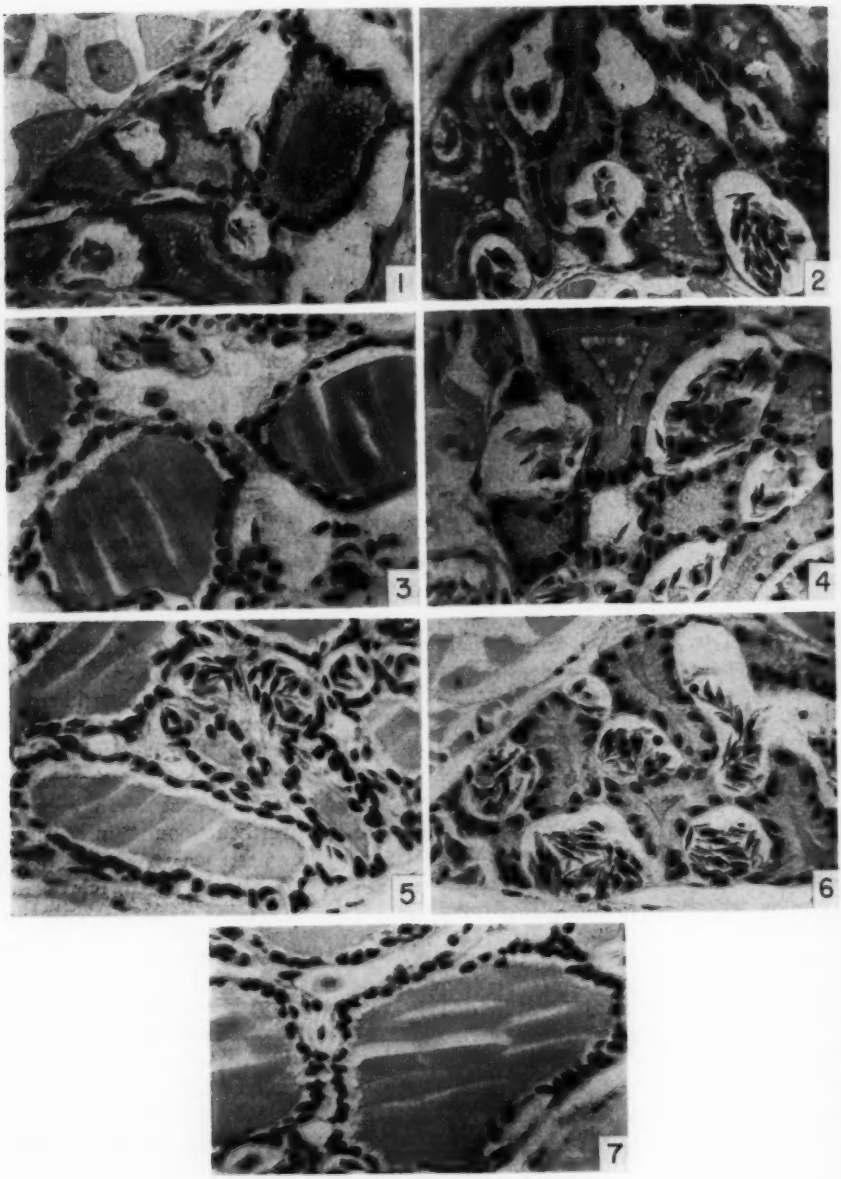
The thyroid glands of the control animals of the 30- and 46-day series did not differ significantly and will be described together. The thyroids of ten *Desmognathus* appeared to have moderate secretory activity. The follicles were relatively large, the colloid was homogeneous, and there was a moderate number of chromophobe droplets. The epithelium was cuboidal to low columnar. There was some but not a great deal of individual variation among the ten specimens. The extremes of variation in epithelial height, follicle size, and vacuolization of the colloid are shown in the photomicrographs (Figs. 1 and 2). The thyroids of ten *Triturus* controls presented a definite contrast with those just described. The follicles were larger, the epithelium much more flattened, and chromophobe droplets were either entirely absent or quite sparse. There was less individual variation in this group than in the *Desmognathus* controls and a single photomicrograph will suffice to illustrate the entire set (Fig. 3).

Examination of five pairs of thyroids of *Desmognathus* given treatment with thiourea for 30 days (15 injections) revealed a definite response indicated by a marked increase in height of the follicular epithelium, a folding of the follicle walls, a reduction in the amount of colloid present, and an increase in the number of chromophobe droplets. The thyroids of animals given this treatment for 46 days (23 injections) showed little difference from those just described except for a further reduction in the amount of colloid. A typical example is shown in Figure 4. The thyroids of *Triturus* given thiourea for 30 days or for 46 days were almost precisely like those of controls. The only definite change is an indication of hyperemia in the glands, the sections showing enlarged capillaries and many more blood corpuscles than were seen in controls (Fig. 5).

Potassium perchlorate treatment produced a still greater effect than thiourea treatment in *Desmognathus*. Even after 30 days, intrafollicular colloid was almost entirely lacking and the increase in epithelial height was quite striking (Fig. 6). Thyroids of animals treated with perchlorate for 46 days showed no significant further change, perhaps indicating that a maximal response had already been elicited. In *Triturus*, perchlorate was no more effective than thiourea in causing histological changes in the thyroid. In fact, there was not even an indication of hyperemia in the glands of the perchlorate-treated specimens; the glands were indistinguishable from those of controls (Fig. 7).

2. *Effect of goitrogens on uptake and release of* I^{131}

Table I gives the compiled results of measurements of radioactivity in thyroids of the animals of the 46-day series in comparison with radioactivity in their heart regions during a 60-hour period after injection with I^{131}. As has been pointed out, the measurements made in the heart region are presumed to be representative of the non-thyroidal tissues in general, except the gut, where iodine was being concentrated and eliminated. The measurements were corrected for the physical decay of I^{131} that followed the injection, and an analysis of variance was made of the means of the corrected counts.



FIGURES 1–7.

The least significant difference beween any pair of means in Table I was found to be 2.63 at the 5% level. The interactions among these individual means are apparently of considerable physiological significance and will be interpreted in the following section of this paper. It is well to note also that the differences between the over-all mean counts for the two species, for the two types of treatment and control maneuvers, for the two locations of counting, and for the six periods of counting were all highly significant in the statistical sense, p being less than 0.1% in each instance.

Discussion

1. General comment and histological findings

The histology of the thyroid gland of *Triturus viridescens* and the seasonal changes it undergoes have been described by Morgan and Fales (1942) who found that the thyroid is moderately active in early winter, gradually increases in activity during mid-winter and spring (up to the breeding season) but has low activity during the summer months. Our *Triturus* controls had thyroids that agreed closely with their description of the summer thyroid characterized by flattened epithelial cells and abundant homogeneous colloid. No comparable study of seasonal varation is available for *Desmognathus*. Our findings showed, however, that there was a definite difference in the histology of the thyroid in these two salamanders at the same season and under the same temperature conditions, *Desmognathus* showing histological indications of a much higher level of activity than *Triturus*.

The experimental use of thiourea as a goitrogenic agent is now well known. Although the precise details of its mode of action are still not completely understood, it is generally accepted that it does not interfere with the ability of the thyroid gland to concentrate iodide but does inhibit its ability to utilize iodide for hormone synthesis (Pitt-Rivers, 1950; Roche and Michel, 1955). As a result of this inhibition, the level of thyroid hormone in the blood of animals treated with thiourea falls, causing increased production of thyroid-stimulating hormone (TSH) by the pituitary. In turn, the elevation of the TSH level induces hypertrophy, hyperplasia, and hyperemia in the thyroid and the release of its intrafollicular colloid. The "goitrogenic" effects of thiourea thus result from the pituitary stimulation rather than from the direct action of the drug itself. In our experiments, the 30- or 46-day treatments of *Desmognathus* with thiourea brought about all these structural changes in the thyroid. *Triturus*, similarly treated, showed no response

Figures 1 and 2. Sections of thyroid glands from control specimens of *Desmognathus* killed in July. Histological evidence of moderate secretory activity ranges from the condition shown in Figure 1 to that shown in Figure 2.

Figure 3. Thyroids of *Triturus* killed in July gave a uniform appearance of inactivity as illustrated in this section.

Figure 4. Section of thyroid gland from *Desmognathus* treated with thiourea. Marked secretory activity is indicated.

Figure 5. Thyroid section from *Triturus* treated with thiourea.

Figures 6 and 7. Thyroid sections from *Desmognathus* (6) and *Triturus* (7) treated with potassium perchlorate. Note marked response shown in *Desmognathus* and lack of response in *Triturus*.

aside from an increase in the vascularity of the gland. This lack of response in *Triturus* agrees with the findings of Adams (1946) who immersed specimens of *Triturus viridescens* in thiourea solutions that were increased in strength over a period of 42 days to 0.528% and then allowed the animals to remain in this concentration for 44 days longer. Such animals showed no change in thyroid histology as compared with controls. Adams found that animals treated similarly with solutions of twice this strength did show an increase in epithelial height and a reduction in colloid, indicating that a sufficiently high dosage may produce a response. Since, however, only two of the ten animals so treated survived to the end of the experiment, it seems clear that this concentration is quite toxic and the changes in thyroid structure may be related to this toxicity. Fisher (1953) reported histological changes in the thyroid of *Desmognathus* after thiourea administration that are in complete accord with our findings. His animals were treated by immersion in a 0.05% solution of thiourea and, although this is only one-tenth as strong as the weakest solution used by Adams, it caused hyperplasia, loss of colloid, and hyperemia—all of which became apparent after only two weeks and had increased markedly by six weeks. Although Fisher's experiments were performed in January and February and the season at which Adams' work was done is not given, the results of these two studies seemed to indicate, as did our findings, that the structure of the thyroid of *Triturus* was relatively unaffected by treatment with thiourea whereas that of *Desmognathus* was markedly altered.

The effects of perchlorate have apparently not been investigated previously for either of these animals. Studies on mammals indicate that the action of perchlorate is quite unlike that of thiourea, for it interferes with the process of iodide concentration (Wyngaarden, Wright and Ways, 1952). The thyroids of animals given effective doses of this drug are unable to accumulate iodine from the plasma. This, of course, prevents synthesis of thyroid hormone (TH) and, just as in thiourea treatment, the resultant lowering of the TH level and rise in TSH level cause the histological changes in the thyroid that are usually associated with a high rate of secretory activity. In our experiments the histological results of perchlorate treatment precisely paralleled those obtained with thiourea. The *Desmognathus* thyroid was strikingly affected but that of *Triturus* remained unchanged.

Desmognathus, then, after treatment with either of these two goitrogenic drugs, gives indication of the increased TSH production, which would be expected on the basis of the work with mammals. *Triturus* shows little or no sign of any increased TSH level. The lack of response in *Triturus* could be explained in at least two different ways. One possibility is that this salamander is refractory to the drugs so that, at the dosage level used, thiourea and perchlorate both failed to inhibit hormone synthesis. If this were the case, the treatment would have caused no lowering of the TH level in the blood; therefore, no stimulation of the pituitary to increased TSH production would have occurred. The other possibility is that the drugs were effective in preventing hormone synthesis but that the pituitary failed to respond in the usual way to the TH decrease. Then no increase in TSH would occur and none of the effects of this hormone on the thyroid would be observed. The results of the study of radioiodine uptake will serve to indicate which of these alternatives is correct.

2. Iodine uptake in control animals

It was observed (Table I) that in the *Desmognathus* controls the counts in the thyroid region were not significantly higher than those in the heart region at 6 hours after injection of I^{131}. By 12 hours, however, they had risen to a high level. They then declined steadily, reaching about one-half the peak level by 2.5 days, but remained significantly higher than the counts in the heart region throughout this period. *Triturus* controls, on the other hand, showed neither an initial difference in counts between thyroid and heart nor a steady decline in the counts of the thyroid region during the 2.5-day period, although the counts at the heart level declined steadily as I^{131} was eliminated from the body, with the result that the thyroid counts were significantly higher than the heart region counts from 24 hours on. Comparison of the thyroid counts with heart counts showed that radioiodine was taken up actively, reached a high level, and was rapidly released by the thyroid of *Desmognathus* whereas in *Triturus* the gland accumulated I^{131} more sluggishly and in lesser amounts and the turnover (during 2.5 days) was negligible. These results are in accord with the histological findings, which indicate a greater physiological activity of the thyroid in untreated *Desmognathus* than in untreated *Triturus*.

3. Iodine uptake in thiourea-treated animals

Table I shows that *Desmognathus* treated with thiourea had a high initial uptake of I^{131} by the thyroid (falling in the same range as the peak for the controls) and then a rapid loss; the differences between thyroid and heart regions were there-

TABLE I

Radioactivity (corrected for physical decay) in counts per second of specimens of Triturus viridescens and Desmognathus fuscus at the indicated periods of time after injection with 5 μc. of I^{131} after 46 days of treatment with goitrogens. Each number represents the mean of counts made on six specimens. The least significant difference (at the 5% level) calculated for these means is 2.63

Species	Time after I^{131} inj. hours	Control animals		Animals treated with thiourea		Animals treated with $KClO_4$	
		Region of thyroid	Region of heart	Region of thyroid	Region of heart	Region of thyroid	Region of heart
Triturus viridescens	6	10.30	10.94	9.64	9.96	10.25	11.68
	12	11.84	9.42	9.90	9.41	8.77	9.85
	24	11.70	8.59	8.90	8.80	5.96	6.22
	36	11.57	7.76	8.86	8.56	5.04	5.03
	48	11.14	7.47	8.79	8.06	3.92	4.06
	60	11.11	7.89	9.10	8.11	3.53	3.95
Desmognathus fuscus	6	8.68	6.80	10.22	6.84	6.40	6.95
	12	12.13	5.66	8.42	6.23	5.58	6.05
	24	10.98	4.56	6.39	4.13	4.67	4.43
	36	10.94	3.78	4.51	3.58	3.91	3.80
	48	8.73	3.64	4.15	3.16	3.14	3.33
	60	6.86	3.35	3.71	3.09	2.84	3.03

fore quite significant at 6 hours after injection of I^{131} but not significant thereafter. In *Triturus,* similarly treated, the iodine content of the thyroid was different from that of the controls only in the 24- and 36-hour counts and the thyroid region showed no count significantly higher than that of the heart region at any time.

These results may be interpreted as follows. It is clear from the histological study that the thiourea treatment caused a hypertrophy of the thyroid in *Desmognathus* and one may assume that this is accompanied by a high affinity for iodine. This would account for the initial high uptake of I^{131}. Since, however, the gland was unable to bind iodine, because of the treatment with thiourea, and also since the TSH level in the blood was presumably high, the I^{131} that had been taken up was very rapidly lost. In *Triturus* the thyroid did not hypertrophy and therefore would not be expected to have a high affinity for iodine. Indeed, it takes up I^{131} and retains it to about the same degree as the controls. Not being able to bind I^{131} the thyroid releases it, but since the TSH level is apparently unaffected by treatment with thiourea in *Triturus,* the release of I^{131} from the thyroid is much slower than in *Desmognathus.*

4. Iodine uptake in animals treated with potassium perchlorate

Treatment with perchlorate produced the same effect on radioiodine uptake in both salamanders. Counts in the thyroid region did not differ from those in the heart region at any time. Indeed, the counts over the thyroid were, in general, slightly lower than those over the heart, probably because of the difference in the volume of circulating blood in the two regions. In any case it seems clear that perchlorate was equally effective in these two animals in preventing any accumulation of I^{131} by the thyroid.

In perchlorate-treated *Triturus,* the counts in the heart region declined more rapidly and reached a significantly lower level than in the control or thiourea-treated *Triturus.* This indicates that elimination of I^{131} from the body goes on rapidly when iodide uptake by the thyroid is blocked but is in some way delayed when the thyroid is actively concentrating iodide. This delay occurs regardless of whether the iodine taken up by the thyroid is ultimately bound to protein. It is well known that, so long as there is no interference with the thyroid's ability to concentrate iodide, the gland acts as an iodine reservoir, continually taking up iodine from the plasma and passing it back at a slow but steady rate so that the decline in iodine content of the plasma caused by elimination from the body is partially compensated by the exchange of iodine with the thyroid. In perchlorate-treated animals, where no such mechanism is in operation, the rate of decline in the iodine content of the plasma would depend solely upon the rate of iodine excretion. It is noteworthy that the rate of decline in the heart-region counts in *Desmognathus* is essentially the same in all three groups. The failure of the thyroid to show any iodine reservoir effect in this animal, even when its iodide-concentrating ability is unimpaired, is probably to be ascribed to the high activity of the gland. In thiourea-treated *Desmognathus,* although the initial uptake of radioiodine was high, I^{131} was very rapidly passed out to the plasma and, since none was bound, the decline in uptake in both thyroid and plasma was rapid, paralleling that of perchlorate-treated *Desmognathus,* where no iodide-concentration occurred at all. It may be assumed that in control *Desmognathus* any unbound

iodine is also rapidly exchanged with the plasma, and the counts in the heart region again fall rapidly. Undoubtedly, the counts in the thyroid region remain significantly high in the controls because in these animals, in contrast with the thiourea-treated specimens, a part of the iodine is bound to protein and is thus unavailable for ready passage into the plasma.

5. Possible adaptive significance of findings

The results of the study of radioiodine uptake indicate that the differences in histological response of the thyroid in *Triturus* and in *Desmognathus* should not be attributed to a difference in the direct effects of the goitrogens on thyroid functioning in the two. Perchlorate treatment definitely prevented thyroidal build-up of I^{131} in both, and there is good reason to conclude that thiourea treatment interfered with binding of I^{131} in both. It would seem, therefore, that the lack of histological change in the *Triturus* thyroid, as well as its normally inactive state, must be attributed to some unusual condition in the pituitary, involving a low level of TSH production. Such an explanation has been suggested to account for what is apparently a similar situation in the goldfish (*Carassius auratus*). The goldfish thyroid exhibits a histological appearance of very low activity throughout the year and at no season (Fortune, 1955) undergoes hypertrophy after administration of thiourea (or other goitrogens). It does, however, respond very strongly to injected TSH both histologically (Gorbman, 1940) and physiologically (Berg and Gorbman, 1954). Fortune (1956) suggests that the inactive thyroid of the goldfish (possibly acquired through many generations of artificial selection) may be an important adaptation permitting its tolerance of an exceptionally wide range of temperatures (0° to 41° C.) and cites the rise in thermal death point from 23° C. to 33° C. in the teleost *Phoxinus laevis* after treatment with thiourea as supporting evidence for his suggestion.

This hypothesis may also apply to *Triturus viridescens*. Although no specific data are available concerning the temperature range of this species, it is undoubtedly quite wide. The animal lives in pools and ponds in both wooded and open situations and has a geographical range from southeastern Canada to Georgia and Alabama. It is certainly able to survive successfully in ice-covered water, and the water temperature in unshaded ponds in mid-summer must frequently rise above 30° C. *Desmognathus*, on the other hand, lives in moist situations usually in close proximity to small streams in deep woods. In our experience it does not survive in the laboratory at temperatures above 25° C. and does best below 20° C. Several rather obvious devices for testing this theory come to mind. One, namely, observation of the effect of TSH administration on iodine metabolism in *Triturus*, is currently being employed by us.

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Summary

1. Specimens of *Triturus viridescens* and *Desmognathus fuscus* were injected on alternate days with 0.1 ml. of 1.0% thiourea. Others were injected on alternate

days with 0.1 ml. of 0.2% potassium perchlorate. Histological study was made of the thyroid glands of both experimental and control animals after 30 days and 46 days of treatment. Measurements of uptake and turnover of injected I^{131} were made on the animals treated for 46 days.

2. Evidence was obtained from the histological observations and from the use of radioiodine to show that although the thyroids of control specimens of *Desmognathus* were physiologically active, those of *Triturus* controls were rather inactive.

3. Both thiourea and potassium perchlorate inhibited thyroidal function in *Desmognathus,* as evidenced by both histological changes and changes in radioiodine uptake.

4. In *Triturus,* thiourea brought about only a slight hyperemia and potassium perchlorate produced no detected histological change in the thyroid. Radiological measurements after the injection of I^{131}, however, indicated that the same physiological responses taking place in *Desmognathus* also occurred in *Triturus* but at a lower level of thyroidal function.

5. Measurements of radioactivity in the heart region demonstrated that iodine was readily excreted from all the specimens of *Desmognathus* and from the individuals of *Triturus* treated with potassium perchlorate. Elimination of iodine was relatively slow in the other specimens of *Triturus.*

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RESPONSE OF THE MALE REPRODUCTIVE SYSTEM OF LIZARDS (ANOLIS CAROLINENSIS) TO UNNATURAL DAY-LENGTHS IN DIFFERENT SEASONS

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In this study we have examined the response of the male reproductive system of the green anole, *Anolis carolinensis,* to unnatural day-lengths at different seasons of the year. Evidence of the photoperiodic control of reproduction in reptiles has been slowly accumulating. Burger (1937) found that artificially increased day-length stimulated a new spermatogenic cycle in the red-eared turtle, *Pseudemys scripta elegans.* Clausen and Poris (1937) reported unseasonal gonadal hypertrophy and spermatogenesis in green anoles exposed daily to 18 hours of light. Bartholomew (1950, 1953) described similar gonadal recrudescence in the desert night lizard, *Xantusia vigilis,* maintained at 16-hour day-lengths.

The above investigators have been primarily interested in the condition of the gonads. Descriptions of accessory sex organs have not been detailed enough to allow for a positive statement that reptiles can be brought into full breeding condition by artificially increasing day-length. In the experiments to be described we have examined both gonads and certain accessory sex organs to obtain a more complete measure of breeding condition. Further, the influence which the condition of the reproductive system at the beginning of an experiment has upon its response to day-length alterations has not been thoroughly examined in all seasons. We have studied the effect of short days prior to and during the breeding season, as well as the effect of long days during and following the breeding season. To our knowledge there has been no previous effort to determine whether a seasonal "refractory period," characteristic of passerine birds (for review see Hammond, 1954), is present in reptiles or if a period of exposure to short days is necessary before a new reproductive cycle can be initiated (Miller, 1954).

Methods

Animals were collected as needed over a period of three years in the vicinity of New Orleans. Adult animals (61 to 71 mm. snout-vent length), that had probably been through a previous reproductive period, were separated from immatures or sub-adults (51 to 60 mm.). The latter had not been through a previous complete reproductive hypertrophy but were due to become sexually mature in the next normal breeding season (Fox, 1958).

Methods of animal care have been reported previously (Fox and Dessauer, 1957). Cage temperatures were maintained at 28 ± 2° C. Artificial lighting was supplied by means of daylight fluorescent lamps mounted above the cages. These were regulated by automatic time switches to supply 18L (hours of light per

24-hour period), 16L, 14L, 9L or 6L. Animals exposed to natural day-length were placed in front of a north-facing window.

Experiments were conducted for approximately 60 days unless otherwise stated. At the end of each experiment lizards were killed with ether, and their fat bodies, liver and left testis weighed. Loss of body weight during the course of an experiment, and liver and fat body weights at autopsy were useful in estimating the degree of starvation of unresponsive lizards. The right testis with attached epididymis, the ductus deferens, and the right kidney were fixed in Bouin's solution, embedded in paraffin, sectioned at 10 micra, and stained with Harris' hematoxylin and eosin. The state of spermatogenesis and the cytology of interstitial cells and accessory sex organs were studied microscopically. Measurements of the diameter of seminiferous tubules, the diameters and epithelial heights of the ductus epididymidis, ductus deferens, and the sexual segment of the kidney were made with a calibrated ocular micrometer. All mensural data were plotted as histograms and subjected to the "*t* test" for possible significance. The size of each sample, mortality and number of starving animals (fat bodies less than 1% of body weight) are presented in Table II. Column "N" in Table II is the number used in calculating standard deviations and "*t*" values for determining the level of significance of the data when it seemed justifiable to eliminate the measurements on starving animals. Differences are accepted as significant at the 5% level and highly significant at the 1% level of probability.

In reporting the results, frequent reference is made to arbitrarily delimited stages of the normal reproductive cycle of wild male *Anolis*. These stages are characterized briefly in Table I; detailed descriptions are presented by Fox (1958).

Although considerable effort was made to locate and measure interstitial cells of the testes, it was felt that these data were unsatisfactory for valid statistical analyses. Data on the sexual segment of the kidney, however, afford an index to androgenic activity (Reynolds, 1943). The sexual segment of *Anolis*, like that of *Sceloporus* (Forbes, 1941), includes the entire set of uriniferous collecting ducts and ureter (Fox, 1958). Measurements were taken in the distal, middle, and proximal regions of the collecting ducts, but only those of the distal end are recorded in Table II.

Results

Experiment 1. September–November: Adult lizards exposed to 18L and natural day-length

At the beginning of the experiment adults had just completed a breeding season and the gonads and accessory sex organs were in their most atrophic state

Table I

Arbitrary stages in the normal reproductive cycle of Anolis

	Spermatogenesis	Accessory sex organs
Stage I	Dividing spermatogonia	Atrophic
Stage II	Primary spermatocytes predominate	Slight hypertrophy
Stage III	Maximum development; large numbers of spermatocytes and spermatids	Near maximum hypertrophy
Stage IV	Large numbers of spermatids; spermatocytes reduced	Maximum hypertrophy
Stage V	Only spermatids numerous	Partial atrophy
Stage VI	Expulsion of all active cells	Atrophic

TABLE II

Experiment	Light exposure	Number	Mortality	Starving	N	Seminiferous tubule diameter μ	Spermatogenic stage						Ductus epididymidis epithelial height μ	Ductus deferens diameter μ	Sexual segment epithelial height μ
							I	II	III	IV	V	VI			
1 Sept.–Nov. Adults	18L	19	2	2	15	120–290† 206±48.9††	2	9	4				11–35† 20.0±6.7††	40–100† 59±15.1††	13–22† 16.1±2.9††
	ND*	14	0	0	14	50–170 110±26.2	14						8–13 10.8±1.5	40–60 53±8.0	13–16 14.0±1.1
2 Sept.–Nov. Immatures	18L	33	12	2	21	90–280 174±57.6	7	9	5				10–30 18.8±6.1	40–140 62±27.2	13–25 17.7±3.6
	ND	16	5	2	11	70–170 107±31.6	11						10–16 13.7±2.1	30–60 48±7.7	13–22 16.9±2.7
3 Oct.–Dec. Immatures	18L	45	9	6	30	90–290 173±62.0		9	15	6			8–32 18.4±8.4	50–180 81 ±38.0	16–38 23.3±5.0
	16L	14	5	3	6	90–260 156±67.5		2	1	3			12–38 21.7±10.5	50–210 120±68.8	16–32 20.4±3.5
	9L	29	9	3	17	80–190 127±29.2	10	7					6–13 11.6±2.1	40–70 54±8.8	13–25 19.1±2.3

* Natural day-length (maximum = 14 hours, minimum = 10 hours).
† Range.
†† Mean and standard deviation.

TABLE II—*Continued*

Experiment	Light exposure	Number	Mortality	Starving	N	Seminiferous tubule diameter μ	Spermatogenic stage						Ductus epididymidis epithelial height μ	Ductus deferens diameter μ	Sexual segment epithelial height μ
							I	II	III	IV	V	VI			
4 Nov.–Jan. Adults	18L	26	5	3	18	110–300 245±50.1	1	1	10	6			12–30 25.6±6.6	90–220 167±45.3	20–50 35.8±9.8
	ND	21	0	8	13	90–280 176±56.1		9	4				10–18 13.1±2.4	30–60 45±12.6	12–20 16.9±2.6
5 Dec.–Feb. Adults	18L	8	4	2	4	240–305 271±31.2			1	3			22–29 24.8±3.4	67–130 106±30.6	17–32 22.5±6.6
	16L	26	6	2	18	240–310 269±15.5			5	13			19–38 27.6±6.6	100–190 150±30.8	25–50 37.6±8.1
	9L	20	0	4	16	230–300 269±24.3		1	11	4			16–35 22.5±4.8	80–230 142±52.4	16–40 24.9±7.6
6 Jan.–March Immatures	18L	26	6	2	18	160–330 256±38.1			3	13	2		21–38 30.0±6.6	120–300 185±49.5	25–51 37.3±8.7
	9L	25	15	3	7	180–300 246±40.9		1	5	1			18–32 22.4±4.9	100–210 142±38.9	21–32 26.0±3.9

TABLE II—*Continued*

Experiment	Light exposure	Number	Mortality	Starving	N	Seminiferous tubule diameter μ	Spermatogenic stage						Ductus epididymidis epithelial height μ	Ductus deferens diameter μ	Sexual segment epithelial height μ
							I	II	III	IV	V	VI			
8 May–July Adults	18L	10	4	0	6	176–300 212±48.3			1	3	2		16–32 24.0±5.4	70–192 149±49.4	13–32 22.0±8.2
	ND	11	2	1	8	210–262 239±17.4			1	7			19–36 28.3±4.9	160–196 179±12.6	22–51 37.0±9.1
	9L	8	4	0	4	77–120 100±18.7						4	9–16 13.4±2.7	50–85 63±15.4	12–17 13.7±1.8
9 July–Sept. Adults	18L	22	3	2	17	112–290 222±40.2	2	3	7	5			13–32 23.9±6.6	77–215 150±42.5	18–51 33.3±10.1
	ND	10	0	0	10	55–118 81±22.1						10	10–20 14.9±3.6	40–96 70±20.1	13–20 15.9±2.5
10 Aug.–Oct. Adults	18L	15	1	3	11	80–250 189±70.1	2	1	4	4			10–35 24.5±8.7	50–230 178±70.7	13–35 22.3±7.9
	14L	28	7	3	18	70–280 146±71.1	5	10	3				10–38 21.2±9.2	60–150 100±21.4	13–26 18.1±4.1
	ND	33	6	0	27	75–135 120±17.9	27						12–23 16.1±2.9	45–75 62±9.5	12–19 15.9±2.5

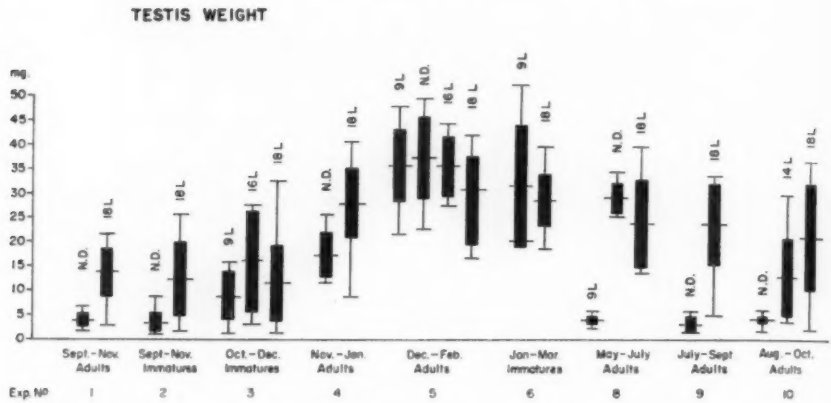

FIGURE 1. Testis weights demonstrating the seasonal gradient in testicular response to exposure to long photoperiods. The terminal horizontal bars indicate the range in sample weights, the middle horizontal bar indicates the sample mean and the solid rectangle represents the standard deviation plotted on both sides of the mean. N. D. = exposure to natural day-lengths; L = hours of artificial light per 24 hours.

(end of Stage VI). By the end of the experiment, animals in the wild had initiated spermatogonial mitoses but their accessory sex organs were still atrophic (Stage I). The 18L sample underwent a highly significant increase in testis weight (Fig. 1) although two apparently healthy animals did not respond to the light treatment. Control animals retained small gonads that averaged significantly less in weight than those of wild animals in November (Fig. 2). Seminiferous tubule diameters were greater and spermatogenesis more advanced in the experimental sample (Table II). Whereas none of the control animals advanced further than spermatogenic Stage I, four of the experimentals were classified as early Stage III. The total cell number, however, was about half that present in a normal Stage III gonad.

The accessory sex organs were partially stimulated by exposure to 18-hour day-lengths at this season (Table II). The ductus epididymidis underwent a very significant increase in diameter and epithelial height. In half of the sample the cells contained secretion granules (Stages II and III); in six animals sperm were present in the lumen (beginning of Stage III). Secretion granules and sperm were lacking in all the controls (Stage I). Neither the average of epithelial height nor diameter of the ductus deferens significantly increased in the 18L sample (Table II). In three animals, however, sperm had entered this organ and it was significantly enlarged, convoluted and contained secretion granules (Stage III). The ductus deferens of the controls remained atrophic (*viz.*, empty, not convoluted, and with a pseudostratified agranular epithelium).

Measurements of the sexual segment (Table II) did not reveal significant differences between the means of the experimental and control samples. Four experimental animals, however, showed slight hypertrophy and the presence of eosinophilic granules at the tips of the epithelial cells of the ureter and the distal

ends of the collecting tubules (Stage II). No hypertrophy or secretion occurred among the controls.

Experiment 2. September–November: Immature lizards exposed to 18L and natural day-length

Although the reproductive organs of these young animals had achieved a partial maturity during the previous summer, they were completely atrophic at the beginning of the experiment. Wild animals in this age group were at minimal reproductive development at the close of the experiment (Stage I).

Immature lizards on 18L underwent a highly significant gonadal response. Testis weights were markedly greater ($P < 1\%$) than the controls (Fig. 1). Four were more responsive to the light treatment than adults during the same period. Spermatogenesis (Table II) almost reached peak activity (Stage III) in a few individuals, whereas none of the controls progressed beyond Stage I.

Of the accessory sex organs studied, only the ductus epididymidis proved to be significantly hypertrophied ($P < 1\%$) by the long light treatment. Over half the animals showed secretory granules and one-third had sperm in the epididymis. In all controls the epididymis was atrophic and without sperm. Three experimental animals showed enlargement of the ductus deferens (Stage II). They had a few sperm in the lumen, the epithelium was taller and the cytoplasm granular. The ductus deferentia of the controls were atrophic (Stage I).

Five experimental animals showed a slight hypertrophy of the sexual segment of the kidney with secretion granules at the tips of epithelial cells near the ureter (Stage II). The sexual segment of a few controls underwent a minor hypertrophy but no signs of secretion were present.

Experiment 3. October-December: Immature lizards exposed to 18L, 16L and 9L

At the beginning of this experiment the animals were in a stage of minimal development. The healthy animals on 16L and 18L showed a highly significant increase in testis weight over those kept at 9L (Fig. 1). Animals that lost weight did not respond to the light treatment. Many kept on the long days had very large seminiferous tubules and had progressed into Stages III and IV of spermatogenic development (Table II), without achieving, however, the high volume of sperm production characteristic of adults during the breeding season. Nearly half of the 9L animals attained the spermatogenic development of adult wild animals for December (Stage II), the others remained in Stage I.

Both samples on 18L and 16L showed highly significant or significant hypertrophy of the various accessory sex organs when compared to those on 9L. The differences between the 18L and 16L samples were not statistically significant. The epididymis of the animals on long days contained many sperm. The cytoplasm of the ductus epididymis was granular and the cell height and diameter much enlarged (Stages II and III). The epididymes of controls were all in Stage I. Significant numbers of animals on long day treatment had sperm in the ductus deferens. In these individuals the diameter of the ductus was increased, the epithelium hypertrophied and the cytoplasm granular (Stage III). A few animals remained in Stage I or II.

The sexual segment underwent a significant hypertrophy on the 18L ($P = 1\%$) and 16L ($P = 5\%$) regimes. Secretion occurred in only five animals on 18L and one on 16L. In these animals the epithelium of the ureter and the distal end of the collecting tubules exhibited eosinophilic granules in the apical ½–⅔ of the cells (Stage II). The 9L series showed only slight hypertrophy, and no signs of secretion (Stage I).

Experiment 4. November–January: Adult lizards exposed to 18L and natural day-length

During November the reproductive system of *Anolis* is in Stage I. Lizards on 18 hours of day-length underwent marked hypertrophy of the testis ($P < 1\%$) compared to controls on natural day-length (Fig. 1). The testes of the controls enlarged also, but the sample mean did not differ significantly from that of wild animals killed in January (Fig. 2).

Most of the experimental animals reached the peak of seminiferous tubule diameter and spermatogenic development (Stage III). A few passed the peak and advanced to Stage IV. Four control animals progressed to early Stage III but these were far from attaining peak development. The remainder of the controls were in spermatogenic Stage II.

The accessory sex organs of the experimentals were greatly hypertrophied ($P < .01$; Table II). In most of the sample the ductus epididymidis was filled with sperm and the epithelial cytoplasm completely filled with eosinophilic granules (Stages III and IV). Four controls had a secretory epididymis with a few sperm in the ductus (Stage II). The ductus deferentia of the 18L sample were very convoluted, filled with sperm and highly secretory (Stages III and IV). Those of the control sample were largely atrophic and empty (Stages I and II).

The sexual segment of the experimentals was hypertrophied (Table II) and highly secretory at its distal end in all animals (Stage II) and in its middle portion in half the sample (Stage III). Control animals showed no signs of secretion although wild animals at this time show slight activity (Stage II).

Experiment 5. December–February: Adult lizards exposed to 18L, 16L and 9L

During this period the reproductive system of wild animals progresses through Stage II with some individuals reaching early Stage III. The distribution and sample means of testis weights were very similar for animals exposed to 9L and 16L (Fig. 1). Testes averaged smaller in the 18L sample. There were no significant differences in seminiferous tubule diameters (Table II). All healthy animals in the 16L and 18L series reached peak spermatogenic development (Stage III), and two-thirds passed on to Stage IV. Animals in the 9L sample were largely in Stage III, but a few merited assignment to Stage IV. When subjected to the Chi-square test, the 16L and 18L samples proved to be very significantly advanced beyond the 9L sample.

In the 16L sample, but not in the 18L sample, the epithelial cells of the ductus epididymidis were significantly taller than those of the 9L sample. Diameters of both the ductus epididymidis and ductus deferens were similar in the 9L and 16L samples, but averaged significantly smaller in the 18L sample. All were secretory

and contained sperm. The ductus deferens appeared to be more highly convoluted in the 16L sample than in the 9L sample, indicating greater sperm storage. Those of the 18L animals were less convoluted and contained considerably fewer sperm than either of the two other samples.

The sexual segment was hypertrophied in both the 9L and 16L samples. In the 16L sample Stage III was reached whereas in the 9L sample the sexual segment remained in Stage II. The distal ends of the collecting tubules were hypertrophied and secretory in both samples. The middle portions of the collecting tubules were secretory in thirteen animals on 16L but in only one on 9L. All sexual segments of the 18L sample were atrophic except that of one animal in which the distal end was secretory.

Data on testes weights were available for a series of captive animals exposed to natural day-length during this period. The mean testis weight was comparable to that found in the 9L and 16L series (Fig. 1).

Experiment 6. January–March: Immature lizards exposed to 18L and 9L

At the beginning of this experiment smaller immature male lizards were in Stage I and larger ones in Stage II. At the end of this experiment the mean testis weight of the 9L sample was greater (Fig. 1), although not significantly so, than the 18L sample. However, animals on 18L were far advanced beyond those on 9L in respect to spermatogenesis (Table II). Most of them appeared to have passed the peak of gonad development (Stage IV).

The ductus epididymidis was secretory and filled with sperm in both samples. Epithelial height of the 18L sample (Table II) was significantly greater. The ductus deferens was secretory, convoluted and filled with sperm in most animals of both samples. Those of the 18L sample had a significantly greater diameter, appeared to be more convoluted and to contain more sperm. In three animals of the 9L sample the ductus deferens was pseudostratified, non-secretory and contained very few or no sperm.

The sexual segment was very significantly hypertrophied in the 18L sample. In over half of this sample it was secretory in the ureter and distal ends of the collecting tubules, and in ⅓ it was secretory through the middle portion (Stage III). Most sexual segments in the 9L sample remained in Stage I; in two animals they were secretory through the middle segment (Stage III) and in one secretion occurred in the ureter and distal end only (Stage II).

Regardless of the length of light exposure, most animals that underwent marked testicular development and hypertrophy of the accessory sex organs were over 55 mm. snout-vent length at the start of the experiment and grew to over 60 mm. during the two months period.

Miscellaneous winter experiments

Data available on a few animals maintained for longer than the standard 60-day period are pertinent to this study. Eight immature lizards kept at 9L from December 10 to April 4 underwent considerable hypertrophy of the reproductive system despite the short day-length. Testes ranged from 8 to 33 mg. with a mean of 22 mg. Spermatogenesis reached Stages III and IV in the larger gonads.

These same individuals had sperm in the ductus epididymidis and ductus deferens, both of which were hypertrophied and secretory. The sexual segment of two larger and faster growing individuals was secretory through the middle portion (Stage III). Those lizards that reached 60 mm. snout-vent length had the largest gonads and the most hypertrophied accessory sex organs. In those less than 60 mm. the extent of testicular hypertrophy was closely correlated with body growth.

Four animals of the same size group survived 18L exposure during the above period. The range in testicular size (3 to 33 mg.) was similar to that in the group exposed to 9L but the gonads averaged considerably smaller (16.5 mg.). Only two possessed large gonads with spermatogenic activity equivalent to Stage IV. One of the latter animals, that had grown more than the rest, had hypertrophied accessory sex organs (Stage III), the other had atrophic accessory sex organs and a spermatic granuloma of the epididymis. The remaining two animals were in spermatogenic Stage VI. The epididymis and ductus deferens were either empty or contained cellular debris. None of the accessory sex organs was secretory (Stage V). Four adult lizards on 9L from January 9 to April 4 were in spermatogenic Stage IV and the accessory sex organs approached Stage III. Similarly, ten adults on 9L from February 3 to April 4 were in spermatogenic Stages III and IV and their accessory sex organs in Stage III.

Experiment 7. April–June: Adult lizards exposed to 6L and natural day-length

On April 30, 26 adult male lizards were placed on a regime of 6L. Five of these animals were sacrificed on June 3 and compared to a series of 24 control animals maintained on natural day-length. Testis weight was very significantly less in the 6L sample (22 to 33 mg; 29.5 ± 3.1 mg.)[1] than in the controls (24 to 52 mg.; 37.0 ± 6.5 mg.), but was larger than that of two samples of wild animals taken in June (Fig. 2). Spermatogenesis in the 6L animals was in late Stage IV typical of wild animals in July, whereas the controls were in spermatogenic Stages III and early IV. The accessory sex organs of the 6L sample were secretory and were not significantly different from those of the controls.

Nine additional animals from the 6L sample were sacrificed on June 21 and compared with a sample of nine newly captured animals. Testis weights were very significantly less (4 to 22 mg.; 14.9 ± 5.5 mg.) than those of wild animals (16 to 33 mg.; 23.7 ± 5.6 mg.). Eight of the 6L animals were in spermatogenic Stage V (typical of August animals) and one in Stage VI. All wild animals were in spermatogenic Stage IV. The epididymis and ductus deferens were significantly reduced in size and in secretory activity in a few experimental animals. All experimental animals showed significant reduction in epithelial height of the sexual segment, but a few retained secretory activity in the distal end of the tubules.

An attempt was made to determine whether the precocious termination of the sexual cycle, which was brought about by exposure to short days, resulted in a temporary gonadal refractoriness to stimulation by long day-lengths. Six animals which had been maintained on 6L from April 30 to June 20, were exposed to 18L from June 21 to September 18. Three responded markedly and three did not. Testis weights (3 to 17 mg.; 10.8 ± 7.2 mg.) were significantly heavier than those of a sample of eight controls maintained on natural day-length from June 4 to

[1] Range; mean and standard deviation.

September 18 (2 to 6 mg.; 3.5 ± 0.6 mg.) and a sample of nine wild animals killed September 22 (1.5 to 4 mg.; 2.3 ± 0.7 mg.). Controls and wild animals were at the end of the annual spermatogenic cycle (Stage VI) and all accessory sex organs were atrophic. Of the six experimental animals two were in spermatogenic Stage VI, one advanced to Stage I, and three advanced to Stage II. The latter three individuals exhibited a precocious spermiogenesis and the epididymis was hypertrophied and contained sperm. The ductus deferens and sexual segment were secretory (Stage II) in only a single specimen in which sperm had reached the ductus deferens. The two unresponsive animals were seriously starved.

Experiment 8. May-July: Adult lizards exposed to 18L, natural day-length and 9L

At the initiation of this experiment the reproductive system of newly captured animals was near peak development (Stage III). During July wild lizards undergo a regression of spermatogenesis (Stage IV), but the accessory sex organs are still near maximum development. The two experimental samples for this period had a relatively high mortality due to starvation (Table II). Spermatogenesis was exhausted (Stage VI) in the 9L sample (Fig. 1) and the accessory sex organs were atrophic (Table II). Considerable variation existed in the 18L sample. Its average testis weight (Fig. 1) was very significantly less than that of controls exposed to natural daylight, but it was nearly equal to the average for wild animals in July. Only one individual was markedly stimulated by the excessive light treatment. Its reproductive organs were at peak activity and comparable to those of an animal in April or May. Except for a slight increase in the number of primary spermatocytes, the gonads of three animals were comparable to those of control or wild animals in July; the sexual segment of the kidney, however, was less hypertrophied. The two remaining lizards had smaller gonads and an atypical spermatogenic pattern. The germinal elements closest to the lumina of the seminiferous tubules were all advanced spermatids (Stage V) whereas the outer layers revealed a proliferation of spermatogonia and primary spermatocytes (Stage I). In one of these animals the ductus deferens and ductus epididymidis were moderately hypertrophied and contained sperm, in the other these organs were atrophic. The sexual segment was inactive in both. Since these animals gained weight, the poor response to light did not appear to be due to starvation.

Experiment 9. July-September: Adult lizards exposed to 18L and natural day-length

The sample means of the experimental animals were very significantly greater for all measurements taken (Fig. 1 and Table II). The testes of the controls were lacking in spermatogenic activity (Stage VI). The testes of the experimentals varied from 5 to 34 mg. and were judged to be in spermatogenic Stages I–IV.

Control and wild animals sacrificed in September have atrophic accessory sex organs. Among animals maintained on 18L, the ductus epididymidis and ductus deferens were highly convoluted, filled with sperm and secretory in twelve; nearly empty and much reduced in three; empty and atrophic in two. The sexual segment of the kidney was hypertrophied and secretory in the ureter and distal ends of the

ducts in six, and non-secretory in the others. The animals that were least responsive to the light treatment possessed fat bodies weighing around 100 mg. (5% of body weight). This represented half or less the weight of fat bodies of animals that yielded a good response.

Experiment 10. August–October: Adult lizards exposed to 18L, 14L, and natural day-length

This experiment was initiated at a time when rapid involution was occurring in all reproductive organs. By the end of the experiment both wild animals and controls had completed a reorganization of these organs and were ready to initiate a new cycle. The testes of most animals in both experimental groups were markedly ($P < 1\%$) enlarged (Fig. 1). Both the maximum and average responses of the 14L sample were considerably less than those of the 18L sample in respect to testis weight and spermatogenic development (Table II). On the basis of a highly convoluted and sperm-packed ductus deferens, four lizards on 18L were classified in spermatogenic Stage IV. The fat bodies of the unresponsive lizards on 18L weighed half as much as those that responded well. Most of the unresponsive animals on 14L had very large fat bodies.

Hypertrophy of the accessory sex organs of the 18L sample tested to be very highly significant except for the sexual segment of the kidney for which $P = .02$. Only the four animals classified in spermatogenic Stage IV showed secretion in the middle and distal portions of the collecting ducts. The hypertrophy of the accessory sex organs of the 14L sample proved to be significant, although no secretion was found in the sexual segment of the kidney.

Discussion

Degree of response of Anolis to artificially lengthened days. In the fall, when gonads are smallest, a 60-day exposure to 18L was sufficient to produce numerous spermatozoa in the larger testes and a precocious, but limited, spermiogenesis in the smaller ones. Although the size of the gonads was only about half that of animals during the breeding season, the relative increases were the greatest of the entire series. Assuming that testes weights were equivalent to those of wild anoles at the beginning of the experiments, gonadal weights of both adults and immatures underwent at least a six-fold increase (Experiments 1 and 2). The closer to the breeding period (April through August) an experiment was started, the more complete was gonadal response. The relative increase in weight, however, was proportionately less.

A similar seasonal gradient in responsiveness was observed in the accessory sex organs. The sexual segment is a more reliable index of this gradient than are the epididymis and vas deferens in which the apparent activity varies with sperm content. Exposure to 18L for 60 days starting in September or October (Experiments 1 to 3) did not bring the sexual segment of adult or immature lizards into a secretory condition typical of the breeding period. However, in Experiment 4, started November 14, half of the adults developed secretory sexual segments typical of April breeding animals. Progressively greater responses were obtained in each succeeding experiment through the winter (Table II).

Our data on the completeness of the testicular response correspond reasonably well with the results of others working with reptiles and birds (for references see reviews by Hammond, 1954 and Farner, 1955). Only rarely do experimental, photoperiodic-stimulated gonads achieve the size that is typical of the particular species during the breeding season; further, the largest gonads are invariably induced just prior to the natural time for hypertrophy. The data of Vaugien (1955) on testis size and bill color of immature English sparrows best illustrate the seasonal gradient of response.

Several factors have been suggested to account for the seasonal variation in the response of the reproductive system. Vaugien (1955) considered the increasing responses which he observed to be due, in part, to the increasing age of the immature sparrows. In immature anoles the degree of response appears to be correlated with growth rate as well as age (as determined by size). Vaugien also demonstrated that in immature English sparrows the longer the exposure to short days prior to capture, the greater the testicular response to long day-lengths. Exposure to a period of short days in the fall is apparently essential for spermatogenesis in a number of species of birds. In adult anoles, exposure to long day-lengths during or following the involution of the testes (Experiments 9, 10, and 1) elicited a response without prior exposure to short photoperiods.

It is generally accepted that the annual variation of the reproductive systems of wild vertebrates is primarily determined by the cyclic nature of pituitary secretions. However, Van Oordt (1956) demonstrated a seasonal difference in the sensitivity of spermatogonia to pituitary gonadotropins in the frog, *Rana temporaria*. On the basis of thyroxin injections, Vaugien (1955) postulated that during short days the testes of immature English sparrows become increasingly susceptible to stimulation. It is possible, however, to explain adequately the data on *Anolis* without assuming a seasonal change in sensitivity on the part of the reproductive system. The gradient of response of the testes can be accounted for by the number of spermatogonia present at the beginning of each experiment, there being fewest in September and progressively more during the winter. The gradient observed in the accessory sex organs appears to depend both upon the state of their respective cells and the state of the interstitial cells at the beginning of each experiment. In September the reproductive organs are atrophic and secretory interstital cells are virtually absent (Fox, 1958). At this time, a long period of stimulus appears necessary since the interstitial cells must be brought into activity before their secretions can secondarily stimulate the accessory sex organs. During later months hypertrophied interstitial cells are increasingly more abundant. These probably immediately release androgenic hormone which, in turn, would bring about progressively greater enlargements of the already partially hypertrophied accessory sex organs.

The above explanation could account for the marked response of some individuals in Experiments 9 and 10. Although the reproductive organs and interstitial cells of these animals were declining at the start of the experiments, they were not atrophic and could respond rapidly to a new stimulus. Conversely, the results of Experiment 7, in which lizards hastened into an early atrophy of the reproductive organs by exposure to 6L responded only mildly to exposure to 18L, could be explained by the degree of atrophy at the time the long day stimulus was applied.

On the basis of data obtained by exposing normal and pinealectomized anoles to normal and long days, Clausen and Poris (1937) have suggested that the pineal eye inhibits the testicular cycle. It is interesting to compare data from our Experiment 4 with their data since the two experiments were conducted at approximately the same time of the year. The testes weights of our animals on either long or short days during this period appear to be nearly identical to those of their pinealectomized animals on similar light regimes. Of the two samples they maintained on normal day-lengths the unoperated controls averaged 0.8 g. less in weight at the beginning of the experiment. The average weight of this sample (3.92 g.) suggests that it was composed largely of sub-adult animals. Since testis weight in *Anolis* has been shown to regress significantly with body size (Fox, 1958), the differences between the two samples could be due to inequalities in sampling. Similarly, in their two samples kept on a long day program, the unoperated animals averaged 0.7 g. lighter than the operated. However, even without this consideration it is doubtful whether the minor difference of 1.5 mg. between the mean testis weights would prove to be statistically significant. In our opinion, the data of Clausen and Poris (1937) do not afford adequate proof that the pineal eye acts as an inhibitor of the male reproductive system in *Anolis*.

Studies on *Anolis* and other species emphasize the importance of examining reproductive structures other than the testes or sperm-storing organs when determining the breeding status of an animal. The presence of viable sperm may not always be a reliable indicator as to whether or not an individual is in full breeding condition. Experiments 1, 2, and 3 indicate that the response of the accessory sex organs may lag behind spermatogenesis. Further, in many species the male normally stores sperm during a non-breeding stage of the reproductive cycle (Fox, 1952; Harrington, 1956). To determine the true breeding status some reliable test for the presence of androgenic secretions should accompany tests for viable sperm. In the English sparrow the color of the bill serves this purpose (Keck, 1933). In many species the emergence of characteristic behavioral patterns or, more directly, the histology of the interstitial cells has been correlated with androgenic activity. We have made only cursory observations on the above criteria in this study. The nuchal crest, a secondary sex character, appeared to enlarge in animals brought into full breeding condition. Although no detailed records of behavior were made, we observed frequent attempted copulations between males exposed to long days in the winter and spring experiments.

The histology of the sexual segment of the kidney in lizards can be used as an accurate measure of androgenic activity and the stage of the breeding cycle. First, cell height and tubule diameter can be measured with precision. Second, the progressive spread of secretion granules can be traced both intracellularly (from the apices to the basal nuclei of the tall columnar cells) and from the ureter and distal ends of the collecting ducts to the proximal collecting ducts.

If the presence of viable sperm alone were used as an indicator it would appear that male anoles were brought into breeding condition by exposure to 18L for 60 days at any season of the year. However, examination of the sexual segment reveals that none were brought into breeding condition in experiments started in September and October and only half of those in experiments started in November.

Refractoriness to photoperiodic stimulation. Passerine birds characteristically

exhibit a refractoriness to stimulus by long photoperiods at the close of the breeding season. This refractoriness persists until there has been a period of exposure to long nights (Wolfson, 1952). The refractory period allows for the physiological reorganization of the gonadal and fat cycles (Wolfson, 1954) and is probably caused by seasonal reduction in pituitary activity (Miller, 1948, 1949; Farner and Mewaldt, 1955).

We have been unable to find a period during which *Anolis* is completely refractory to photoperiodic stimulation. The testes seem to respond, to some extent, at all seasons of the year (Fig. 1, Table II). In all experiments except Nos. 5 and 6, which were started near the peak of testicular development (Stage III), a longer photoperiod resulted in either greater mean or maximum testis weights. In Experiments 5 and 6, although there was no quantitative increase, there was a premature progression to a more advanced stage of spermatogenesis. Experiments 8, 9, and 10, initiated after the peak of testicular size had been achieved, yielded variable results. Normally, animals at this time should have progressed from spermatogenic Stages III or IV towards Stages V or VI with the number of dividing spermatogonia and spermatocytes rapidly decreasing. In some individuals, however, all classes of germinal cells were well represented so that the gonad appeared to be in Stage III. In other individuals spermatogenesis was maintained at about a Stage IV level without showing the expected decline. In still others, germinal elements characteristic of Stages I and V were present in the same seminiferous tubule, although intervening stages were missing. This suggests a premature beginning of a new cycle before the completion of the old.

The sexual segment of the kidney appeared to be refractive in experiments started in September and only mildly responsive in October. Although this could be due to a difference in the cyclic nature of the two pituitary gonadotropins as suggested by Farner and Mewaldt (1955), we believe that the poor response of the sexual segment can be accounted for by the delay in arousing the atrophic interstitial cells.

Inherent rhythm. In studying the natural reproductive cycle of male anoles, Fox (1958) noted that spermatogenesis is initiated in the fall and makes considerable progress during the winter despite the short day-lengths. We sought to determine if there was any period during the year in which constant exposure to short days would disrupt the normal cycle. Nine hours of artificial light were chosen instead of 10 (day-length at time of the winter solstice at New Orleans) since 10 hours of bright light obviously are not available during the winter. Nine hours did not disrupt the cycle of immature lizards exposed from October through December and from January through March, nor that of adults exposed from December through February. The accessory sex organs likewise were not retarded by the short days. In fact, in adults of the 9L sample killed in February, both the testes and accessory sex organs were more advanced than those of wild lizards at that date. At the time of writing, additional data were furnished us by Anthony Dimaggio of our Biochemistry Department. Six immature males (49–57 mm.), maintained at 6L for 60 days ending March 1, appeared to have normally active testes (left testis ranged 12–40 mg., mean = 24.3 ± 11.4 mg.). One must conclude that reasonably short day-lengths are not very effective in disrupting the inherent reproductive rhythm of male anoles in the fall, winter and early spring when gametogenesis is on the upswing.

Fox (1958) also stated that the peak of spermatogenesis for most individuals of this species was achieved in April. Normally, all maintained quite active spermatogenesis through July. Experiment 7 showed that six-hour day-lengths, initiated at the end of April, produced a significant reduction in testis weight within 34 days, and highly significant atrophy in both the testes and accessory sex organs within 52 days. Nine hours of light (Experiment 8) from May to July also precipitated complete involution. Thus, it appears that short days will end a reproductive cycle prematurely after the cycle has neared or passed maximum development.

The stimulating effects of different day-lengths. Since our original choice of 18 hours for a long day stimulus was arbitrary, we designed three experiments to test whether shorter periods might be equally stimulating but have fewer detrimental effects upon the animals. In Experiment 3 all average measurements of the 16L sample tended to be greater than the 18L sample. However, the larger gonads and more secretory sexual segments occurred in the 18L sample. In Experiment 5 a few adults on 16L gave a better response than any on 18L. The smaller gonads of the 18L sample were judged on a histological basis to be more advanced than those of the controls, but there does not appear to be justification to similarly appraise the smaller accessory sex organs. We believe the data for the 18L sample reflect the exhausting features of long hours of wakefulness imposed upon animals with initially low fat reserves, rather than a lack of stimulation. Experiment 10 demonstrated that exposure to 14L from August to October was stimulating (Fig. 1) to the testes but had little effect on the accessory sex organs. On the other hand, 18L was very stimulating to both gonads and accessory sex organs.

These data indicate that, in general, the longer the day-length the greater the response of the reproductive organs of *Anolis*. A similar relationship between day-length and gonadal size of the white-crowned sparrow has been thoroughly analyzed recently by Farner and Wilson (1957). Eighteen-hour day-lengths appeared detrimental to many anoles (see mortalities and starvation, Table II). For this reason a 16-hour day-length is probably more satisfactory to use as a long day stimulus for *Anolis*. Fourteen-hour day-lengths (the maximum day-length for New Orleans) are definitely stimulatory to *Anolis*, but the response may not be sufficiently rapid to be detected in short-term experiments. Dessauer (1953) found no differences in the metabolism of anoles exposed for three weeks to 10L and 14L. It now seems likely, in view of the considerable individual variation he obtained, that three weeks may not have been sufficient or the light regimes not sufficiently different to produce marked contrasts in metabolism.

The response of immature lizards compared to adults. The usefulness of immature anoles as experimental animals was impaired by their high mortality rate which resulted from starvation. However, since they were more abundant than adults in the wild it was easier to collect a large series. They responded to the long day-length exposure very much as did the adults without any indication of a refractory period. Comparison of the September experiments (I and II) indicates that the non-starving immature lizards responded as well as or better than the adults. Likewise, the maximal measurements recorded in Experiment 6 (Table II, Fig. 1) compare favorably with the best results on adults in any experiment. The most striking responses occurred in animals above 55 mm. (snout-vent

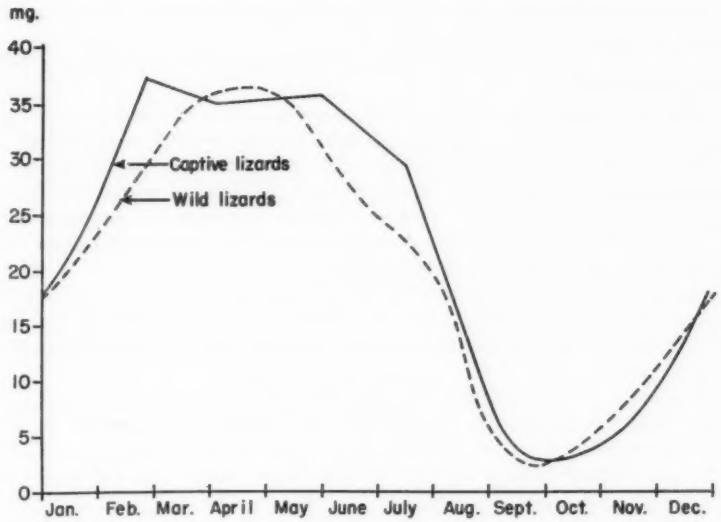

FIGURE 2. Curves comparing the annual variation in testis weights of freshly captured wild anoles (Fox, 1958) with those of laboratory controls exposed to natural day-lengths for 60 days.

length). All individuals with markedly active reproductive organs grew at least several millimeters and usually reached 60 mm. in snout-vent length.

Data on animals maintained for periods longer than two months suggest that unseasonal long photoperiods are most stimulating during the first two months. Over a period of four months the controls matched the earlier achievements of the experimentals and the experimentals regressed.

Captive lizards exposed to natural day-lengths compared to wild lizards. The marked weight difference between testes of two-month captive lizards exposed to natural day-lengths and wild lizards can be seen in Figure 2. From February through most of July the testes of captive controls usually averaged significantly heavier than those of wild animals sacrificed at approximately the same date. Bartholomew (1950) found similar differences in captive and wild yucca night lizards (*Xantusia vigilis*). He suggested that the differences were due to the higher temperature of the laboratory. In the case of *Anolis*, this could easily account for the differences during the winter and spring but it is doubtful whether it would account for the differences in June and July.

SUMMARY

1. The reproductive system of adult male anoles was stimulated by artificially lengthened days at all seasons of the year. At least a few males were brought into full breeding condition in experiments initiated from November through August. Sperm were produced at all seasons.

2. A refractory period, such as occurs in passerine birds, did not appear to be present in *Anolis*.

3. A previous exposure to short days was not essential for obtaining a response to a long photoperiod.

4. Both the testes and accessory sex organs exhibited progressively greater responses in successive experiments through the fall, winter, and spring.

5. The seasonal gradient of response which produced increasingly larger testes could be accounted for by the increasingly larger number of spermatogonia at the beginning of each experiment. The gradient in response of the accessory sex organs was dependent on the normal cyclic fluctuations of their histology and the abundance of hypertrophied interstitial cells.

6. The inherent rhythms of both the gonad and accessory sex organs were not disturbed by 60 days' exposure to 9- or 6-hour photoperiods during the fall and winter. However, such exposures near or after the peak of spermatogenesis resulted in premature atrophy of all reproductive organs.

7. Comparison of the effects of 14-, 16-, and 18-hour day-lengths suggested that the longer the day-length the more rapid the response. Extremely long days, however, appeared to have detrimental effects upon some individuals.

8. Most immature lizards of less than 55 mm. snout-vent length responded poorly to the experimental conditions. All that responded well were growing rapidly. Those that grew to 60 mm. or more had reproductive organs as large or larger than older adults.

9. Captive lizards exposed to natural day-length for 60 days at any time between February and July tended to have larger testes than wild lizards sacrificed on the same date.

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A PERSISTENT DIURNAL RHYTHM OF LUMINESCENCE IN GONYAULAX POLYEDRA[1]

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The photosynthetic marine dinoflagellate, *Gonyaulax polyedra*, emits a brief flash of light (duration, about 90 milliseconds) when stimulated by agitation. It is one of the many organisms responsible for the luminescent display sometimes observed in the ocean at night when the water is disturbed (see Harvey, 1952). Previous studies with this organism (Haxo and Sweeney, 1955; Sweeney and Hastings, 1957a) have shown that the luminescent response to stimulation varies rhythmically in a diurnal fashion. Cultures grown in natural illumination, or in artificial lights with alternating light and dark periods of 12 hours each (= LD), display a much greater luminescence during the dark period (Fig. 2).

When LD cultures are transferred to a dark chamber, the rhythm continues but its amplitude decreases progressively. By action spectra studies, it has been found (Sweeney, Haxo and Hastings, unpublished data) that this decrease in amplitude arises from the need for light in the organic nutrition of *Gonyaulax*, via photosynthesis. This finding prompted the search for constant environmental conditions under which the endogenous rhythm would persist, without the loss of amplitude which occurs in continuous darkness.

The possibility of maintaining the cells heterotrophically was explored, but the consistently negative results obtained indicated that *Gonyaulax* is an obligate photo-auxotroph. Continuous bright light inhibits the rhythmic fluctuations in luminescence, and it has not been possible to separate, by using light of different colors, the photosynthetic requirements for light from the inhibitory action of light on rhythmicity. It has been found, however, that if LD cultures are placed in a continuous dim light, the rhythm of luminescence persists without loss of amplitude. It has thus been possible to investigate in some detail the nature of this endogenous rhythm.

Materials and Methods

G. polyedra has been maintained in a modified sea water medium described previously (Sweeney and Hastings, 1957a). The growth rate is dependent upon light, temperature, and the concentrations of mineral nutrients. The maximum growth rate which we have measured is one division per day, but under the condi-

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tions used in the experiments to be described the rates were always less than this. The illumination was provided by "cool white" fluorescent lamps, the intensity being measured in foot-candles with a Weston illumination meter.

The experimental procedure was as follows: stock cultures were maintained in Fernbach flasks containing 1500 ml. of medium. In preparation for an experiment, 2-ml. aliquots from these cultures were pipetted into each of several hundred test tubes at cell densities between 2000 and 7500 cells per ml. All tubes were then subjected to the appropriate conditions of light and temperature. To measure the luminescence at any given time, two tubes were removed, assayed, and then discarded. The cells were stimulated to luminesce by bubbling air through the cell suspension, and the resulting phototube current was accumulated on a capacitor. Luminescence is expressed in terms of the total amount of light emitted during one minute of stimulation, at the end of which time essentially all luminescence has ceased. Additional details of the light measurement procedure may be found elsewhere (Sweeney and Hastings, 1957a).

Results

Demonstration of the persistent rhythm. A persistent rhythm of luminescence may be observed if cells which have been kept for a time under LD conditions are transferred to continuous dim light (about 100 foot-candles). A typical example of the persistent rhythm under conditions of constant light and constant temperature is shown in Figure 5. In similar experiments, we have continued measurements for as long as 14 days; the rhythmic pattern continues undamped during this time. At the light intensity used in such experiments there was little growth.

The natural period of the rhythm. The period of the rhythm is measured by the time between successive maxima in luminescence. When the cells are subjected to alternating light and dark periods on a daily (24-hour) schedule, the period of the rhythm is 24 hours (Fig. 2). Under conditions of constant illumination, however, the rhythmic changes have a period which is close to, but not necessarily exactly 24 hours. Pittendrigh and Bruce (1957) have referred to this as the *natural period*, or the innate period of an endogenous rhythm when light and temperature are held constant.

The natural period in *Gonyaulax* is a function of at least two environmental factors, light intensity and temperature. The effect of light intensity upon the period is illustrated in Figure 1. Cells were placed in continuous light at three different intensities, and it is evident that the natural period was shorter at higher intensities. These experiments also illustrate the light intensity dependence of the inhibitory effect of continuous illumination upon the rhythm. At the two higher light intensities the amplitude of the rhythm was progressively damped, while at the lowest light intensity no marked damping of the amplitude of the rhythm was evident.

The effect of temperature upon the natural period is not large but, contrary to expectation, the period becomes longer rather than shorter as the temperature is raised (Hastings and Sweeney, 1957b). At 16° C. the period was found to be 22.8 hours while at 26.7° C. it was 26.5 hours. A Q_{10} of less than 1.0 is unusual, and the results were interpreted as evidence for a compensation mechanism which functions to keep the period approximately temperature-independent.

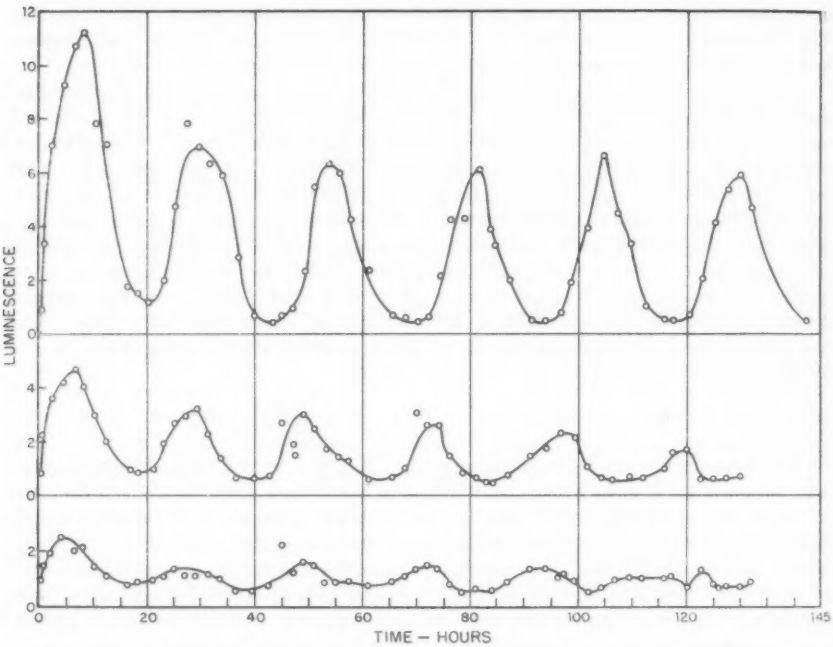

FIGURE 1. The effect of light intensity upon the natural period at constant temperature (21° C.). The cells were grown in LD conditions (800 foot-candles during the light period). The beginning of the experiment, shown on the graph as 0 time, fell at the end of a normal light period. At this time, some cells were placed in the dark, and others in light of 120 foot-candles (upper curve), 380 foot-candles (middle) and 680 foot-candles (bottom). The average periods were as follows: 680 foot-candles, 22.0 hours; 380 foot-candles, 22.8 hours; 120 foot-candles, 24.5 hours; dark, 24.5 hours (not shown on graph; one period measured).

In view of the relatively small temperature effect, the period of this rhythm may be characterized as essentially temperature-independent.

The endogenous nature of the diurnal rhythm. The persistence of the rhythm of luminescence under conditions of constant temperature and light intensity indicates that the mechanism of the rhythmicity is endogenous. Several other experiments serve to support this conclusion.

Figure 2 illustrates one of many experiments in which the phase of the rhythm was shifted by changing the time at which the light and dark periods occurred. In such experiments the phase (*i.e.*, the solar time at which the maximum in luminescence occurs) may be shifted so that it will bear any desired relationship to the solar day. In cultures which are subsequently transferred to constant conditions of dim light or darkness, the phase of the persistent rhythm is related to the previous light and dark program rather than to solar time, or any other factor. Changes in the phase of the endogenous rhythm have not been observed when light and temperatures were held constant.

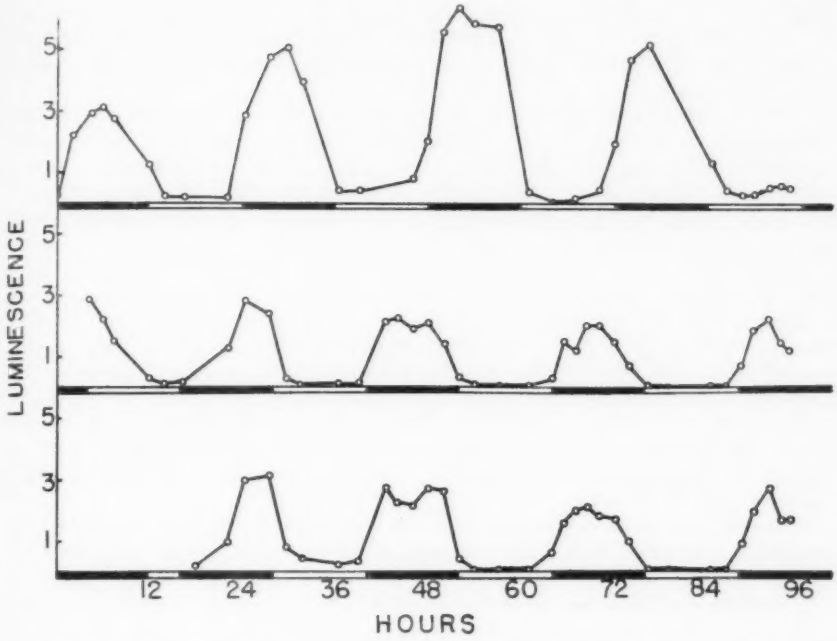

FIGURE 2. This experiment illustrates the effect of changing the solar time at which the light and dark periods occur. The upper curve shows the pattern of luminescence changes in an LD culture which had been on the schedule indicated for some time. The black bars on the time axis indicate dark periods. The lower two graphs illustrate the effect of imposing upon cultures (which were previously on the schedule shown in the top graph) an LD schedule in which the light and dark periods were at a different time of day. The new schedules were started at zero hours on the graph. Temperature, about 26° C. Light intensities used, about 250 foot-candles.

A series of experiments has been carried out from which it is evident that pre-treatment with diurnal light and dark periods (*i.e.*, one dark plus one light period equals 24 hours) is not necessary in order to demonstrate an endogenous rhythm. That is to say, there is no evidence that a "learning" or "memory" process is involved. For example, cells have been exposed to "non-diurnal" light and dark periods which together add up to greater or less than 24 hours, followed by conditions of either constant light or constant dark. An experiment of this sort is shown in Figure 3. In this experiment, cells were exposed to alternating light and dark periods of 7 hours each for about 100 hours. During this period the luminescence changes were quite evidently governed by these light and dark periods so that there was a maximum in luminescence every 14 hours. At the end of this treatment, some cells were placed in constant dim light and others in darkness. In both cases a diurnal rhythm with a period of approximately 24 hours was evident. The 14-hour cycle had not been "learned," even though it had been

possible to entrain the luminescence rhythm to the 14-hour cycle. A difference between those placed in darkness and those in dim light was that the amplitude of the rhythm in darkness progressively decreased as a result of the lack of light (see introduction).

Similar experiments have been carried out in which the alternating light and dark periods were 6 hours each, 8 hours each, and 16 hours each, giving cycles of 12, 16 and 32 hours, respectively. The results were similar to those shown in Figure 3. After about 100 hours of such a non-diurnal light-dark cycle the cells were placed in constant dim light and a rhythm of luminescence having a period close to 24 hours was evident.

Another series of experiments has shown that it is not necessary to pre-treat the cells with any sort of alternating light and dark periods in order to demonstrate endogenous diurnal rhythmicity. As mentioned previously, if cells are grown in continuous bright light (*ca.* 800–1500 foot-candles) there is no detectible rhythmicity. Cells maintained in this way for several months, or for as long as several years, have been found to exhibit a diurnal rhythmicity when they are placed in darkness (Haxo and Sweeney, 1955; Sweeney and Hastings, 1957a). The phase of the rhythm which is initiated when the cells are moved from bright light to darkness is independent of the solar time, and related only to the time at which the light-to-dark transition is made.

A similar result was obtained when cells which had been grown in bright light for almost one year were merely transferred to dim light. This experiment is

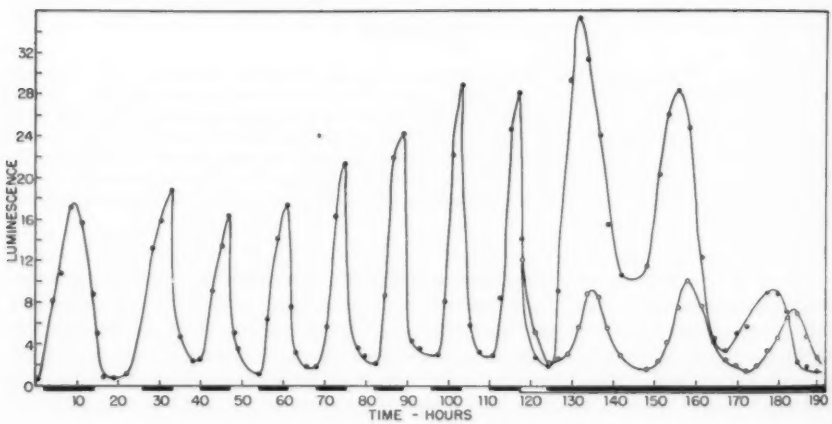

FIGURE 3. This illustrates the entrainment of the luminescence rhythm to a 14-hour cycle and the manifestation of an endogenous diurnal rhythm when the cells are placed in constant conditions subsequent to the treatment. Dark periods are indicated by black bars on the time axis. The cells were on an LD schedule previous to the time when the 14-hour cycle was started (at 26 hours). Light intensity throughout the 14-hour cycling was 800 foot-candles. At 117 hours some aliquots were removed from the dark and placed in constant light at 230 foot-candles. The luminescence changes in these cultures are shown by the circles. From 124 hours on, the other aliquots were left in the dark and the luminescence changes are plotted with solid dots. Temperature, 21° C.

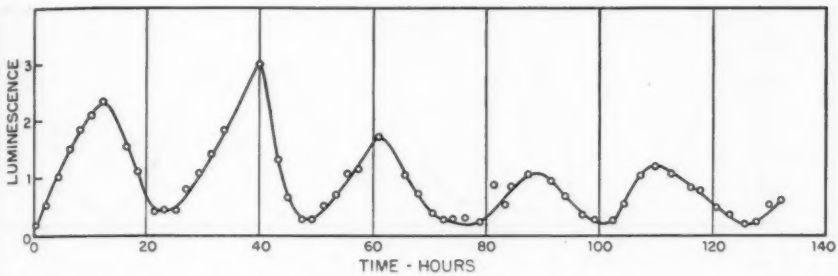

FIGURE 4. The initiation of an endogenous diurnal rhythm of luminescence by means of a one-step change in illumination. Cultures which had been grown in bright light for one year were moved from bright light (800 foot-candles) to dim light (90 foot-candles) at the time indicated on the graph as 0 hours. Luminescence measurements were made approximately every two hours thereafter. Temperature, 21.0° C. Average period, 24.5 hours.

illustrated in Figure 4. It differs from the previously mentioned experiment (in which cultures were moved from bright light to darkness) in that the amplitude does not decrease with time, since light is available for the nutrition of the cells. The precise phase relationship to the time of transfer from bright light is somewhat different, but here also it is not related to solar time.

Phase shift by light perturbation. It is clear from Figure 2 that the phase of the rhythm may readily be shifted by an appropriate manipulation of the light and dark periods to which the cells are exposed. It is not necessary, however, to expose the cells to a new light-dark cycle in order to reset the phase of the rhythm. A single exposure to a different light intensity can result in a stable phase shift. Pittendrigh and Bruce (1957) have discussed the significance of phase resetting of biological rhythms by single light perturbations. If rhythmicity results from an innate oscillatory mechanism characterized by its own natural period, and the phase (but not the period) is determined by the sequence of light and darkness, then it is to be expected that non-repeated light changes should suffice to change the phase. The perturbation therefore need not contain any information concerning period.

The experiment shown in Figure 4 illustrates phase setting by a single step-type light perturbation. The phase of the previously aperiodic cells was determined by the time at which the light intensity was changed. The shifting of phase in already rhythmic cultures is evident in the experiments shown in Figure 3. The entrainment of the rhythm to a 14-hour cycle may be explained by assuming that each transition, either from darkness to light or from light to darkness, serves to shift the phase, so that repetitive phase resetting occurs.

A phase shift in the *Gonyaulax* rhythm by single light perturbations has also been demonstrated in other ways. Figure 5 illustrates a shift in the phase of rhythmic cells which were given a single exposure to either bright light or darkness. The phase shift which results in such experiments has been found to be stable since, in experiments where measurements were continued for an additional 48 hours, the phase difference between the controls and the treated cells remained unchanged.

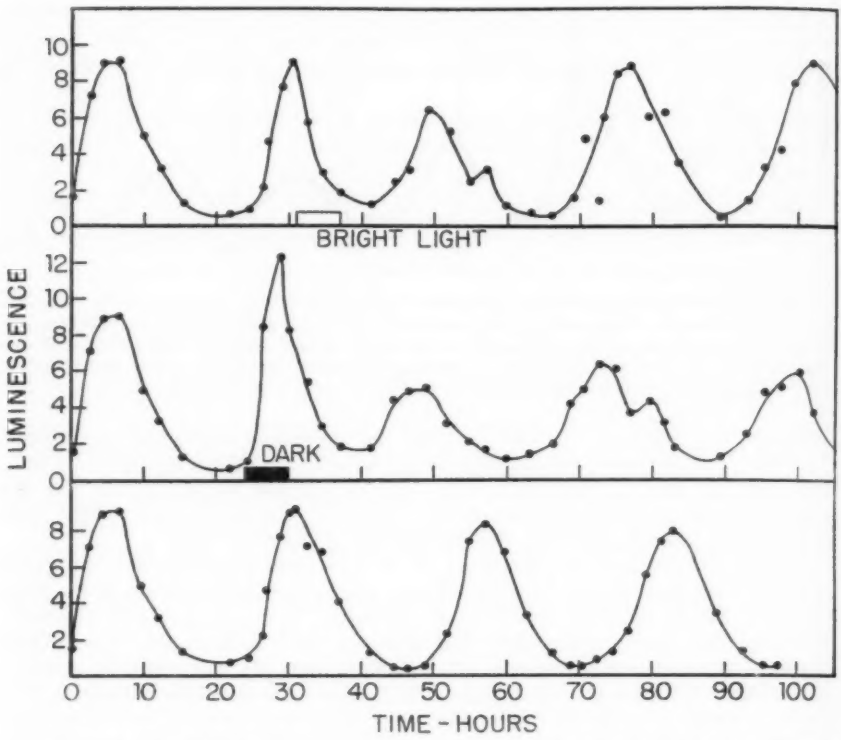

FIGURE 5. This experiment illustrates a phase shift in the rhythm following changes in light intensity. Cells previously kept under LD conditions were placed at constant temperature (23.5° C.) and constant light intensity (100 foot-candles) at the end of a 12-hour dark period. Two days later (zero time on the graph) measurements of luminescence were begun and the endogenous rhythm was apparent. Some cultures (upper curve) were transferred to bright light (1400 foot-candles) for a period of 6 hours and then returned to the previous condition (100 foot-candles). Other cultures (middle curve) were transferred to darkness for 6 hours and returned to dim light at 200 foot-candles. The time at which treatment was given is indicated by bars on the time axis. In both cases a marked phase shift in the rhythm is evident. The control (lower curve) was left in dim light all the while. Average period in control: 25.7 hours.

Figure 6 shows another technique which has been used in the study of phase shifting by single perturbations. Rhythmic cells were placed in the dark and, at a later time, received an exposure to light. Although the amplitude of the rhythm decreases over the next few days, the times at which maxima in luminescence occur are evident, so that the phase may be determined. The number of hours by which the phase is shifted would be expected to be some function of both the magnitude of the perturbation, and the time in the old cycle at which it is administered. The technique of interrupting darkness by light has been used to investigate these parameters.

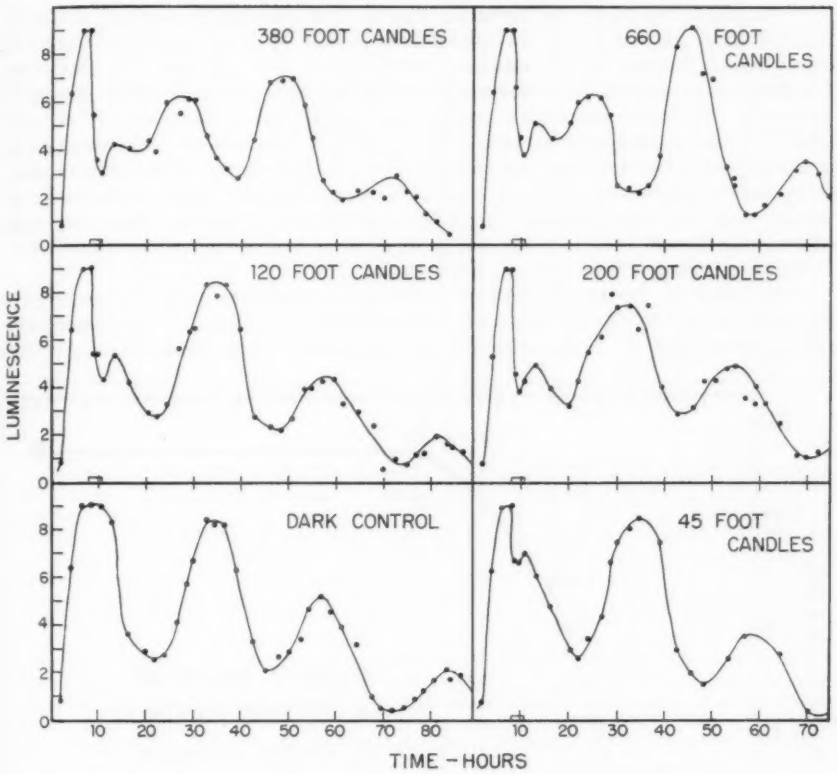

FIGURE 6. This illustrates phase shifting in a rhythmic culture by a single 2½-hour exposure to light, and the effect of intensity upon the magnitude of the phase shift. Prior to the time shown on the graph, all cultures were in LD conditions, and "two hours" on the time axis was the end of the last 12-hour light period. All cultures were put in the dark at that time and the control was left in the dark thereafter. The remaining cells were exposed to a 2½-hour illumination beginning 6 hours after the light-to-dark transition (indicated by the rectangle on the time axis). Following this 2½-hour illumination they were returned to darkness for the remaining time. The intensities used are shown in the figure. A 2½-hour exposure to 1400 foot-candles (not plotted) was found to be no more effective than the exposure to 660 foot-candles (Fig. 7). Temperature during experiment, 21° C.

The effect of varying the light intensity was determined in experiments such as the one shown in Figure 6. The amount of phase shift was found to increase with increasing light intensities, up to a "saturation" value of about 800 foot-candles. This relationship is illustrated in Figure 7, and the stability of the resetting is shown by plotting on the same graph the phase shift measured at each of the subsequent cycles. Several experiments of this sort have been carried out and the same type of relationship has been observed. The quantitative values obtained in separate experiments were somewhat different, however, and the reason for this variation has not been determined.

The magnitude of the perturbation may also be changed by varying the duration of light exposure. In an experiment similar to that shown in Figure 6, the duration instead of the intensity was varied. All exposures (at 800 foot-candles) were started simultaneously, six hours after the cells were placed in darkness. A longer exposure to such a light perturbation was found to be more effective than a shorter exposure. The amount of phase shift was found to be proportional to the duration of the exposure, up to a maximum phase shift of about 11½ hours, which was achieved with 2½ hours exposure. The relationship between phase shift and duration might be expected to be different, depending upon the time in the old cycle at which the perturbations were given, as discussed below. This aspect has not been studied, however.

The effect of varying the time in the cycle at which the perturbation is given has been studied by again using a procedure similar to that used in the experiments shown in Figure 6. Cells grown in LD conditions were transferred to a dark

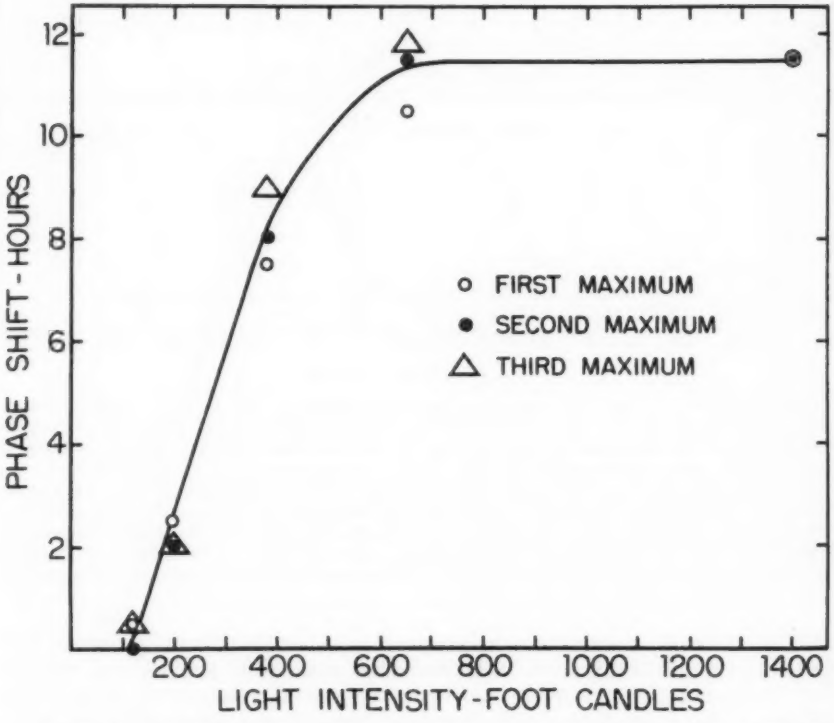

FIGURE 7. The relationship between the intensity of a single 2½-hour light perturbation and the number of hours by which the phase is shifted. Data taken from the experiments shown in Figure 6. Different symbols, as marked on the graph, give the phase difference between the control and the experimentals, measured at each of the three maxima in luminescence subsequent to the perturbation.

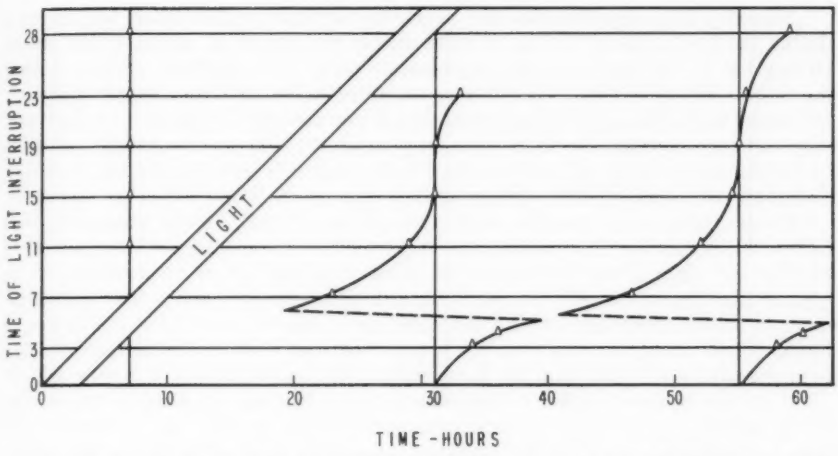

FIGURE 8. The effect of light perturbations (1400 foot-candles for 3 hours) given at different times during the cycle, upon the phase of the endogenous rhythm. Cells which had been kept under LD conditions were placed in the dark at zero time on the graph, which was the end of a 12-hour light period. The times at which the maxima in luminescence occurred in the control, which remained in the dark all the while, are indicated by vertical lines. In the experimentals, a triangular symbol shows a time at which a maximum in luminescence occurred, and thus represents phase. The experiments were carried out in a way similar to those illustrated in Figure 6. Each horizontal line represents a different experiment. For example, the line at 11 hours on the ordinate was an experiment in which a maximum in luminescence occurred at 7 hours. A light perturbation was begun at 11 hours and terminated at 14 hours. Maxima in luminescence occurred subsequently at 29½ hours and 52½ hours. The other experiments are represented in a similar way. The relationship between the time in the cycle at which the light perturbation was administered and the number of hours by which the phase was changed may be better visualized by rotating the figure by 90°.

chamber at the end of a light period. At regular time intervals thereafter, some of the cells were removed and exposed for three hours to light at an intensity of 1400 foot-candles, and then returned to darkness. Times at which exposures to light were made were selected so that the experiment served to scan somewhat more than a full 24-hour cycle. A control received no exposure to light, and the times at which maxima in luminescence occurred in this control are indicated by the vertical lines in Figure 8.

The results of the experiments are summarized in Figure 8. First of all, it may be noted that the new phase, following a light perturbation, was not directly related to the time at which the light perturbation was administered. That is, the maxima in luminescence did not occur at a fixed time interval following the light treatment. If that had been the case, the symbols indicating phase would fall along a line at 45°, parallel to the lines representing the times at which light exposures occurred. This latter type of result was obtained in experiments mentioned previously (Figure 4, for example) where a rhythm was initiated in an arrhythmic culture, and the phase was determined only by the time at which the light intensity was changed.

Secondly, it is apparent that the sensitivity to light perturbations was greater during the first 12 hours (Fig. 8) than during the second 12 hours of the cycle. During the first 12 hours a rather pronounced phase shift resulted, whereas during the second 12 hours there was little or no phase shift. In other longer term experiments it has been found that this variation in sensitivity continues in a rhythmic way. It may therefore be stated that, in general, the cells are maximally sensitive to a light perturbation at a time when luminescence is near maximum, and that this sensitivity declines to a minimum at a time when luminescence is minimum.

Finally, however, it may be noted from Figure 8 that a light exposure given before the maximum in luminescence results in a phase delay, so that the time between the light perturbation and the subsequent maximum in luminescence is greater than 24 hours. On the other hand, a light exposure given after the maximum in luminescence results in a phase advance, such that the next maximum in luminescence occurs in less than 24 hours. This difference is illustrated by the light perturbations which start at three hours and at seven hours in Figure 8.

Perturbation by mechanical stimulation. It is of interest to consider the nature of the cellular component or components which, being modified as a result of the light perturbation, result in the observed phase shift. If perturbation by means other than light also resulted in a change in the components of the rhythmic mechanism, then a phase shift would be similarly expected. It seemed possible that mechanical stimulation might be effective in this regard. Consequently, a perturbation experiment was carried out, in which air was bubbled through the cell suspensions instead of exposing the cells to light (Fig. 9). No phase shift occurred; the cells which had been stimulated retained the same phase as the unstimulated controls.

The experiment also shows that it is possible to modify the concentrations of compounds which are involved in the luminescence rhythm without having any effect upon the phase of the rhythmic mechanism itself. It was found previously (Hastings and Sweeney, 1957a) that the rhythm of luminescence involves a daily variation in the amount of extractable components of the luminescent system (luciferin and luciferase). Mechanical stimulation causes the luminescent reaction to occur, so that one would suppose that the concentrations of components in the luminescent system (and other biochemical systems coupled to it) might be changed. In fact, the apparent effect of stimulation is similar to the effect of light; the luminescence decreases to a low level in both cases. But since no phase shift occurred following stimulation, it does not seem likely that the luminescent system could be directly involved in the basic rhythmic mechanism, although it is clearly coupled to such a mechanism. Moreover, it is evident that there is no feedback from the luminescent system to the system controlling the phase of the rhythm. From previous evidence we had suggested that the luminescent system might itself constitute an autonomous chemical oscillation (Hastings and Sweeney, 1957b). The results described above, however, favor a hypothesis which proposes a basic mechanism of cellular rhythmicity to which various physiological and biochemical processes, such as luminescence or cell division (Sweeney and Hastings, 1957b; 1958), could be coupled.

Cellular interaction. Since all the experiments which have been described are carried out with large cell populations (4000–15,000 cells per tube), the ques-

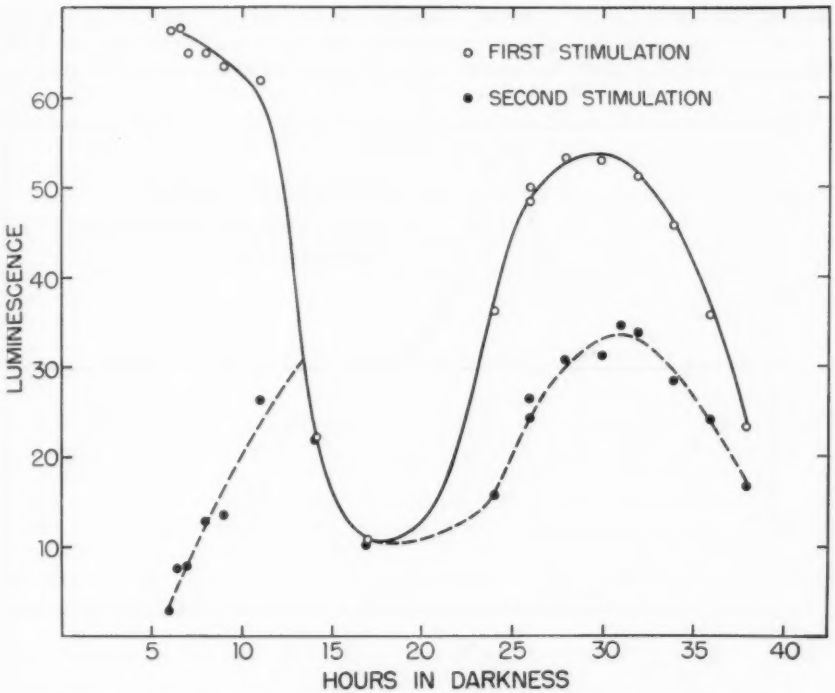

FIGURE 9. The effect of perturbation by mechanical stimulation upon the phase of the rhythm. Previous to the time shown, all cultures were in LD conditions, and zero time on the graph was the end of a light period. At this time all aliquots were placed in the dark. Six hours later a large number of aliquots were stimulated by bubbling air, but were not exposed to light. The luminescence changes of both these and the controls were determined through the subsequent maximum in luminescence. No significant change in the phase of the stimulated cultures was observed.

tion arises as to whether or not some cellular interaction might occur. Since the rhythmic mechanism involves fluctuations in the concentrations of chemical components within the cells, it is conceivable that certain diffusible compounds might escape into the medium, and that their concentrations might also fluctuate in a diurnal fashion. The importance of such a phenomenon would be evident if the supposed compound or compounds could function, as in a feedback mechanism, for stabilizing the frequency and/or phase of the rhythm. It is also possible that some other phenomenon, such as cellular motility, could be involved in such a feedback mechanism. This latter possibility seems unlikely, however, in view of the fact that mechanical stimulation, with its attendant violent motion and disturbance of cellular motility, did not result in a phase change.

An experiment in which this question was investigated is illustrated in Figure 10. Two cultures were maintained under LD conditions for several weeks with

their phases different by 5 hours. Samples were pipetted from each culture and moved to constant dim light at the end of a dark period. After each had been under constant conditions for several days (their phases still being different by

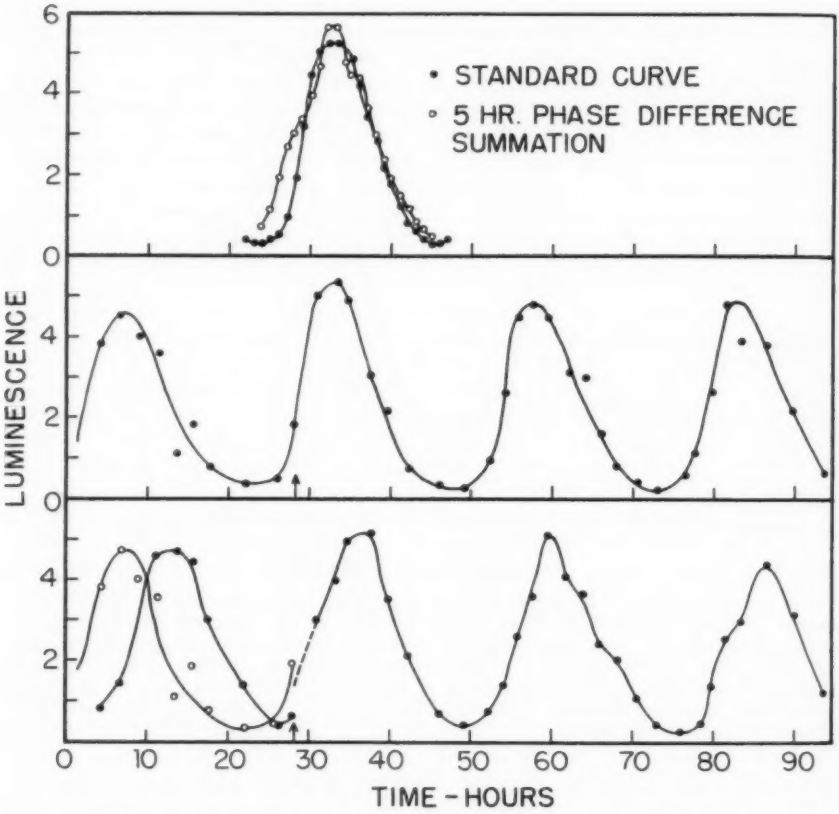

FIGURE 10. The effect of mixing two rhythmic cultures which were out of phase with one another. Cultures which had been in constant dim light for several days, having a 5-hour phase difference as shown (bottom curves), were mixed at the time indicated by the arrow. The rhythm continued, with a phase having its maximum at a time precisely halfway between the maxima of the two original cultures. The middle curve shows the result of mixing cultures having the same phase, done at the time indicated by the arrow. No change of phase was observed. The upper graph shows the result which would be theoretically expected upon mixing two cultures 5 hours out of phase, on the assumption that no interaction was involved. Two "standard" luminescence curves, which were measured from a culture which had not been mixed, were summated with a 5-hour phase difference. For purposes of comparison, the resultant curve is plotted on the graph along with the original standard curve, the latter having been displaced by 2½ hours on the time axis and normalized to the calculated curve. It may be seen that the shape of the calculated curve does not differ greatly from that of the original "standard" luminescence curve.

5 hours), the cultures were mixed in equal proportions, and the luminescence changes in the mixed cultures were measured.

If two typical curves showing the luminescence rhythm are summated, the phase of the two curves being different by five hours (75°), the resultant curve differs only slightly in shape from the original curves (Fig. 10, top). The maximum of the resultant curve lies precisely midway between the maxima of the two original curves.

In the actual mixing experiment, the maximum in luminescence of the mixed cultures occurred halfway between the maxima of the two separate unmixed cultures. Moreover, the shape of the curve from the mixed cultures was very similar to that which was obtained when the measured luminescence of the separate cultures was summated. The mixing experiment therefore indicates that no cellular interaction was involved.

Discussion

The subject of persistent endogenous rhythms has been recently reviewed by Harker (1958), Pittendrigh and Bruce (1957), and Bünning (1956). These reviewers, as well as other authors, have taken the view that the property of rhythmicity may be a nearly universal feature of organisms. This view is derived, largely, from the observation that endogenous rhythms are extremely widespread, having been reported from a large variety of both plants and animals. Furthermore, Pittendrigh and Bruce develop the generalization that most, if not all organisms can measure time; that they possess clocks. They consider that the basic mechanism evolved early, and that it has been retained in the course of evolution as a part of the adaptive organization of all organisms. Their use of the word "clock" refers to the basic mechanism involved in cellular rhythmicity, and the essential properties of this mechanism are considered to be similar in different organisms.

Pittendrigh and Bruce (1957) thus distinguish between the clock as the basic mechanism, and the persistent rhythms which are presumed to be controlled by the clock. Other authors (Brown, Hines, Webb and Fingerman, 1950; Stephens, 1957a; Harker, 1958) have similarly concluded that an overt persistent rhythm may be distinguished from an underlying mechanism, and our studies with *Gonyaulax* give support to this thesis. For example, since it was found that concentrations of compounds taking part in the luminescent reaction could be changed without shifting the phase of the rhythm, it is probable that the luminescence rhythm does not in itself constitute the basic mechanism. Furthermore, we have recently reported a persistent rhythm of cell division in *Gonyaulax* (Sweeney and Hastings, 1957b). The luminescence rhythm and the cell division rhythm have essentially identical properties. Moreover, we have not been able to demonstrate a phase shift in one rhythm which is not accompanied by a similar phase shift in the other rhythm. These findings give additional support to the hypothesis that one basic mechanism controls both rhythms.

The identity and physico-chemical nature of the presumed basic clock mechanism in persistent rhythms remains undefined. But if the properties of this basic mechanism in *Gonyaulax* may be deduced from the rhythm of luminescence, then it is evident that the mechanism possesses essential clock-like properties; the

period is not greatly affected by environmental factors, but the phase is labile to resetting by the appropriate external changes. We may note, in addition, that light emission in *Gonyaulax* is clocked so that it is maximal during the night phase, when it is visible; and without environmental inhibition, luminescence is minimal during the day phase. However, since the possible utility of the light emission is not known, the functional significance of clocked luminescence is not apparent.

Many of the characteristics of the rhythm of luminescence which we have described are similar to the characteristics of persistent rhythms in a variety of other organisms, ranging from other unicellular forms to mammals. The comparisons outlined below do not pretend to be complete, but they serve to illustrate the point. The remarkable similarities found support the view of Pittendrigh and Bruce (1957), that the basic mechanism involved in rhythmicity is the same in all organisms.

Practically all the persistent diurnal rhythms described have natural periods which are close to but different from 24 hours. This includes rhythms in *Drosophila* (Pittendrigh, 1954), *Uca* (Webb, Brown and Sandeen, 1954), *Oedogonium* (Bühnemann, 1955a), *Euglena* (Bruce and Pittendrigh, 1956), and many others. The natural period may range, in different organisms, from about 21 to 27 hours. In fact, significant differences in the natural periods in different individual mice are well documented (Pittendrigh and Bruce, 1957).

Studies of rhythms in a variety of organisms, including the bee (Wahl, 1932), *Uca* (Brown and Webb, 1948), *Avena* (Ball and Dyke, 1954), *Drosophila* (Pittendrigh, 1954), and *Euglena* (Bruce and Pittendrigh, 1956), have shown that in each case the period is nearly the same at temperatures which differ by 15° C., or more. It is interesting to note that the effect of temperature upon the period of the *Gonyaulax* rhythm is similar to that reported by Bühnemann (1955b) for the rhythm of sporulation in *Oedogonium*, in that the apparent Q_{10} for both is less than 1.0. Two cases may therefore be interpreted as the result of an over-compensation in the mechanism responsible for temperature independence (Hastings and Sweeney, 1957b).

Only a few experiments have been specifically designed to detect the effect of different light intensities upon the natural period of persistent rhythms. In those cases which have been reported (see Harker, 1958), the natural period has been found to change no more than an hour or two under different light intensities.

The entrainment of rhythms to periods different from 24 hours has been reported in several organisms, including *Euglena* (Bruce and Pittendrigh, 1956) and *Oedogonium* (Bühnemann, 1955a). In these and other cases, as in *Gonyaulax*, the rhythms return to the characteristic natural period when the organisms are returned to constant conditions.

On the other hand, several experiments have been reported in which rhythmic organisms still continue to show a 24-hour rhythm while being subjected to light-dark cycles which differ from 24 hours. For example, Webb (1950) found that the period of the *Uca* rhythm was not changed while the organisms were subjected to light (95 foot-candles) and dark periods of 16 hours each, and Tribukait (1954) found that entrainment to an imposed light-dark cycle occurred in the mouse only so long as the imposed cycles did not differ greatly from the natural period.

Studies with *Gonyaulax* suggest a possible reason for the lack of apparent entrainment in experiments such as those cited above: the light intensities used may not have been sufficiently bright. In *Gonyaulax*, the luminescence rhythm may be entrained to periods which differ greatly from the natural period. Our interpretation of this entrainment is that repetitive phase resetting results in a period corresponding to the imposed schedule. The importance of light intensity as a parameter in phase shifting by single light perturbations has been documented in experiments with *Gonyaulax*. That it is equally important in entrainment has been shown in an experiment with *Gonyaulax* described elsewhere (Hastings and Sweeney, 1958), in which it was found that entrainment occurred at a light intensity of 800 foot-candles, but not at 200 foot-candles.

Entrainment of rhythms to imposed cycles which are only slightly longer or shorter than the natural period has been discussed by Pittendrigh and Bruce (1957). Their interpretation suggests that the mechanism may be different from that involved in entrainment to cycles differing greatly from the natural period.

The role of 24-hour light-dark cycles in establishing the phase of diurnal rhythms has long been recognized, and experiments with many organisms have demonstrated that, as in *Gonyaulax*, the phase shifts in response to a new light-dark cycle which is out of phase with solar night and day. The fact that the light intensity used in such experiments is of importance has been shown by Brown, Fingerman and Hines (1954).

That non-repeated light perturbations are capable of establishing or changing the phase of a persistent rhythm has been stated as an important generalization only in recent years (Pittendrigh and Bruce, 1957), although some previous studies (Kalmus, 1940; Webb, 1950) do provide examples of the phenomenon. The phenomenon provides another analogy between the characteristics of persistent rhythms and the known properties of physical oscillators. It is well known that a single disturbance or perturbation applied to an oscillating system will quite generally shift its phase without any modification to the period, and the behavior of a simple pendulum is a good example. Pittendrigh and Bruce (1957) have found phase shifts following single light perturbations in persistent rhythms of *Euglena* and *Drosophila*, and the rhythm in *Gonyaulax* provides another example of the phenomenon.

Detailed studies on the effect of the duration and intensity of single perturbations have not yet been reported in other organisms, but it appears that the nature of the phase shift in *Gonyaulax* may differ in one respect from that reported for *Drosophila* (Pittendrigh and Bruce, 1957). Following a single light perturbation in *Drosophila* there may occur "transients," so that the phase is not reset immediately but comes to its stable position only after several cycles. In *Gonyaulax*, on the other hand, phase has been found to be reset immediately. The reason for this difference is not known, but it may be related to the relative complexity of the organisms involved.

With respect to the phenomenon of phase shifting, Bruce and Pittendrigh (1957) have discussed whether the resetting signal is the step-up in light intensity (dawn) or the step-down in light intensity (dusk). Several experiments with *Gonyaulax* have adequately illustrated that the phase is labile to both, so that

neither event may be said to be the timing cue to the exclusion of the other. For example, the experiments shown in Figure 3 illustrate both a light-to-dark transition followed by constant darkness, and a dark-to-light transition followed by constant light. In both cases, the last transition resulted in a phase shift.

The action spectrum for shifting the phase of the luminescence rhythm by a single light perturbation shows relatively sharp maxima in effectiveness at 475 mμ and 650 mμ (Hastings and Sweeney, unpublished). The red maximum, in particular, suggests that chlorophyll acts as a photosensitizer for phase shifting. Since the effects of single light perturbations are essentially the same in plants and animals, we may conclude that in *Gonyaulax* the photosensitizers involved in phase determination are not a part of the basic mechanism of rhythmicity. In animals, also, the photoreceptor pigments of the eye are not a part of the basic mechanism, although they function in phase determination by light. Whitaker (1940) reported that blinded mice possess a natural period of about 24 hours in their activity rhythm, but that the rhythm could not be entrained by 24-hour light-dark cycles to correspond with solar night and day, as in normal mice.

It is known that temperature changes (Pittendrigh, 1954; Stephens, 1957a), and perhaps certain other factors (Harker, 1958) may also serve to establish or reset phase. There is no report, however, that mechanical disturbances can be effective in other organisms in this regard.

The possibility that individuals in a population may entrain each other was suggested by Pittendrigh and Bruce (1957). However, Stephens (1957b) was unable to demonstrate any significant phase modification in individual fiddler crabs when they were placed together with crabs possessing a different phase. A similar result was found in the present studies with *Gonyaulax*.

It is of interest to note that the shape of the luminescence curve obtained in experiments where *Gonyaulax* cultures possessing different phases were mixed is not greatly different from that for the unmixed cultures. Indeed, as already pointed out, this is the expected result of adding two luminescence curves which are five hours out of phase with one another. Thus, a population composed of cells having at least two different phases is difficult to distinguish from the usual experimental populations, in which we have assumed that all cells possess the same phase. This experiment serves to caution us. In a biological rhythm having a sinusoidal shape, measurements from populations may not accurately represent the behavior of individual cells.

We do not know how the luminescence of the individual *Gonyaulax* cell at different times in the cycle compares with that measured in a population. The question is an important one, and there are several possibilities which, in the absence of any relevant data, need not be discussed here. This problem is being investigated utilizing measurements of the rhythm of cell division, where the performance of an individual cell may be repeatedly and relatively easily scored.

Although several suggestions have been made concerning the physico-chemical nature of the basic mechanism involved in persistent diurnal rhythmicity (Pittendrigh and Bruce, 1957; Hastings and Sweeney, 1958), none has received any substantial support. It is hoped that information concerning the extent and kind of biochemical changes associated with the rhythms will be of value in understanding this basic problem. Studies of this nature are in progress with *Gonyaulax*.

Summary

1. The characteristics of a persistent diurnal rhythm of luminescence in the dinoflagellate *Gonyaulax polyedra* are described.

2. The light emission upon stimulation, from cultures which are kept in alternating light and dark periods of 12 hours each (= LD), is 40 to 60 times greater during the dark period than during the light period. If LD cultures are placed in continuous dim light (100 foot-candles) a diurnal rhythm of luminescence persists. If LD cultures are placed in continuous bright light (> 1500 foot-candles) the rhythm is damped, and no fluctuations occur in the amount of light emitted.

3. The occurrence of rhythmicity is not dependent upon prior exposure to LD conditions. Cultures which have been grown in bright light for as long as one year show a diurnal rhythm when placed in constant dim light or darkness. Cultures kept in alternating light and dark cycles which are greater or less than 24 hours similarly show a diurnal rhythm when returned to constant dim light or darkness. "Training" or "memory" is therefore not involved.

4. The rhythm can be entrained by light-dark cycles which are different from 24 hours. The period of the luminescence rhythm corresponds to light-dark cycles which have periods ranging between 12 and 32 hours.

5. The period of the rhythm is always close to 24 hours when the cells are kept under constant conditions, but it varies slightly depending upon the temperature and light intensity.

6. The phase of the rhythm under constant conditions is related to the time at which the previous light and dark periods occurred. Moreover, the phase may be shifted by interposing a non-repeated exposure to a different light intensity. The number of hours by which the phase is shifted in such an experiment is dependent upon the intensity and duration of the light treatment, and the time in the cycle when it is administered.

7. Exhaustive mechanical stimulation does not alter the phase of the rhythm.

8. When cultures having different phases were mixed, no evidence was found which would indicate that there was any interaction between them.

9. The evidence presented indicates that the diurnal rhythmicity is the consequence of a basic oscillatory mechanism which is inherent to the cell.

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THE ACTION OF INSULIN ON CELLS AND PROTOPLASM [1]

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In a lecture he gave in 1947, Best (1948) stated, "We often hear the statement made that we have had insulin for 25 years and still do not know exactly how it acts. This is quite true, but we know more about the action of insulin than about any other hormone."

Strangely enough, the great science of endocrinology, with its vast body of information concerning the chemistry and the ultimate effects of various hormones, has not been able to solve the basic problem of why the different hormones act as they do. In recent years in the attempt to understand the action of insulin, there has been more and more emphasis on studies of the cell as a whole rather than on studies of fragments or extracts of cells. In a thoughtful review of the literature, Ross (1956) is led to comment (p. 364), "It is apparent that no consistent effect of insulin has been demonstrated in cell-free systems." And the biochemist Levine, a leading authority in the field of carbohydrate metabolism and long a student of insulin, wrote recently (Levine and Goldstein, 1955) (p. 344), "It would be expected therefore that a certain degree of morphological intactness is necessary to demonstrate hormonal effects and actions. Otherwise we would be pulling the trigger of an unassembled gun." Levine himself has turned to the ways of thought and the methods of cell physiology in order to obtain a solution of the insulin problem.

Thus it may not be too presumptuous for cell physiologists to express ideas about the mechanism of insulin action. Indeed back in 1914, Höber suggested that diabetes might be due to a change in the permeability of cells for sugar. This idea was then taken up by Wiechmann (1924, 1926), and by Häusler and Loewi (1925; see also Loewi, 1927). However, the older evidence in favor of the permeability theory was not very convincing, and interpretations in terms of the cell as a whole were pushed into the background by chemical work which seemed to show that insulin had a specific effect on certain isolated enzyme systems. More recently, and on the basis of newer evidence, the permeability theory has been revived and modernized, and it is finding wide support.

No attempt will be made here to review the enormous mass of literature on insulin. Books on endocrinology contain a great deal of information and there have been a number of recent reviews by authorities in the field (Haugaard and Marsh, 1953; Stadie, 1954; Levine and Goldstein, 1955; Weil-Malherbe, 1955; Ross, 1956; Stich and Maske, 1956).

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A few facts stand out. Some of these have been long known and were mentioned in a review published by Macleod in 1924. For our purposes, it may be well to remind the reader that:

1. The action of insulin in reducing the concentration of sugar in the blood is not due to an action on the blood itself, for insulin does not have this effect on blood withdrawn from the body. Hence the cells and the protoplasm they contain must play a part in the lowering of sugar concentration in the blood.
2. The convulsive action caused by excess insulin is not due to an effect on the cerebrum, for it occurs in decerebrate animals.
3. Lowering of the sugar concentration in the medium surrounding isolated nerve or muscle has no effect in stimulating either nerve or muscle.
4. When a rabbit is given a lethal dose of insulin, violent convulsions occur; these are then followed by a comatose stage, and convulsions and coma continue in alternate phases until, after an hour or more, the animal dies.
5. The harmful results of excess insulin can be relieved by the injection into the blood stream of a small amount of glucose.
6. Lack of insulin causes a failure of fat, carbohydrate and protein formation. Best (1953) sums up the situation (p. 434) by stating that "insulin is a central anabolic hormone without which many of the building processes . . . cannot proceed at the physiologic rate."
7. Insulin also causes an increase in the rate of oxidation of sugar. Thus it is a catabolic hormone as well as an anabolic hormone.

It is clear that insulin markedly increases the rate of activity of various enzymic actions, and yet when purified preparations of these enzymes have been tested, there seems to be little or no effect of insulin upon them. Moreover it would be hard to explain how it would be possible for a single substance to have a direct effect on all the various enzymes responsible for the synthesis of carbohydrates, proteins and fats, as well as those responsible for the oxidation of sugar. Hence we apparently must conclude that in one way or another some change in the cell or its protoplasm has an accelerating effect on many types of enzyme activity.

At the present time, what is doubtless the leading theory of insulin action holds that the primary effect of insulin is to change the cell in such a way as to facilitate the passage into it of various sugars. This theory, due in its present form to Levine and his collaborators, has been supported not only by the work of Levine and his group, but also by the careful and ingenious experiments of various other investigators. Perhaps the most impressive work is that of Park, Bornstein and Post (1955). The entire subject is ably reviewed by Ross (1956), and this review should be consulted by anyone interested in details or references to the rather extensive literature. The permeability theory has been enthusiastically endorsed by Stadie (1957), who is certainly one of the outstanding investigators in the field.

Scarcely anyone has attempted to criticize the permeability theory, although such criticism is possible, both from the standpoint of our knowledge of permeability and transport mechanisms, and also because the theory can scarcely offer an explanation of some of the basic known facts of insulin action. This latter point will be discussed in a later section.

Effect of Insulin on the Permeability to Glucose

Our experiments with insulin were done entirely on relatively simple, isolated cells of lower organisms. These cells offer exceptionally favorable material for the cell physiologist, but work with such cells is open to the criticism that the action of insulin may be confined to the cells of vertebrate animals. Such an opinion is held by Ross in the review cited above, and it was expressed forcibly by Best, Jephcott and Scott (1932). However, there are reports of insulin action on the cells or tissues of protozoa, sponges, flatworms, crabs and insects; on yeast cells, and on various kinds of bacteria. Whether this literature is sound or not is a question we do not care to discuss. Certainly there can be no objection to our using simple living cells to explain insulin action, for if we can obtain effects on these cells with insulin and if such effects can be used to interpret the action of insulin in higher animals, we may be able to offer suggestions of some value.

For the study of cells and protoplasm the eggs of marine invertebrates offer many advantages and this type of material has often been used by cell physiologists. We used the eggs of the sea urchin *Arbacia punctulata,* the surf clam *Spisula solidissima,* and the annelid worm *Chaetopterus pergamentaceus.* All of these eggs are readily available at Woods Hole. If insulin directly favors the entrance of glucose into cells (quite apart from any indirect effect it might have as a result of the utilization or combination of glucose within the cell), we thought that perhaps we might be able to find evidence for such a direct effect on marine egg material. This we were unable to do, and our results were wholly negative. For this reason we shall not attempt to report them in any detail, but will merely cite a few of our experiments as briefly as possible.

One of the standard ways of determining the ease with which dissolved substances pass through cell membranes is to study the osmotic behavior of cells in relation to solutions of the substances in question. There are a variety of such osmotic methods. One of the simplest of these methods depends on the fact that the more readily a substance penetrates, the less osmotic pressure it can exert against the plasma membrane of the cell. By observing the changes in volume of the cells when they are immersed in various concentrations of a given substance, one can obtain a rapid measure of the ease with which a substance enters. In our particular case, if insulin favored the entrance of glucose, a solution of glucose containing insulin would be less potent osmotically than a similar control solution which differed only in lacking insulin. In order to obtain as great an effect as possible, we used saturated solutions of insulin. These were obtained by dissolving 0.2 mg. of insulin in one ml. of the glucose solution. Actually not all of the insulin went into solution. All the solutions were brought to the pH of sea water. The insulin we used was a preparation which was relatively zinc-free; it was kindly supplied by the Eli Lilly Company through the kindness and courtesy of Dr. G. H. A. Clowes. In view of the fact that many types of protoplasm are very sensitive to zinc, we were indeed fortunate to obtain this preparation from which 98.8% of the zinc ordinarily present in crystalline insulin had been removed. Actually an assay made by the Eli Lilly Company showed only 0.0061% zinc in the dry material.

Table I shows the results of our experiments on sea urchin eggs. In this table, as also in Tables II and III, the ± sign indicates standard deviation. A molar solution of glucose caused a slight decrease in the volume of the eggs. In the

TABLE I

Effect of glucose and glucose + insulin on the osmotic behavior of Arbacia eggs. Measurements of diameters were made after the eggs had been immersed in the solutions for 5 minutes. The values show the average of 10 measurements

Diameter of eggs in sea water	77.8 ± 2.02 microns
Diameter of eggs in molar glucose solution	71.5 ± 2.18 microns
Diameter of eggs in molar glucose solution containing insulin	70.7 ± 2.29 microns

presence of insulin approximately the same decrease occurred. If insulin had favored the entrance of the glucose, then the solution of glucose containing the insulin should not have caused as great a shrinkage.

Similar results were obtained with eggs of the clam *Spisula*, as is shown in Table II. Again there is no indication that insulin favors the entrance of glucose.

In our experiments with eggs of the worm *Chaetopterus*, we ran into difficulty. When we immersed these eggs in solutions of glucose, the sugar entered rapidly, so rapidly in fact that even solutions are strong as 2 *M* caused no shrinkage of the eggs. We experimented with 2 *M* and 1.75 *M* and 1.5 *M* glucose solutions with and without insulin. In all cases, in the absence of insulin the glucose entered more rapidly than when it was present. In the 1.75 *M* and 1.5 *M* glucose, frequently the eggs swelled so rapidly that they broke. This sometimes made measurements uncertain. Apparently in the glucose solutions, absence of ions like calcium changed the semipermeable membrane of the cell in such a way that it became permeable to glucose. Perhaps the small amount of zinc in the insulin helped partially to stabilize the membrane. Because of the increased permeability of the cell membrane in the absence of the salts of sea water, we decided to compare the behavior of glucose and glucose + insulin in the presence of an appreciable amount of sea water. The results are shown in Table III. In this case also, presence of insulin does not favor the entrance of glucose.

Our results with marine eggs lead to the conclusion that the effect of insulin in increasing the rate of entrance of glucose into cells is not a general phenomenon true for all types of living material. Other authors in the past have reached the same conclusion. Thus it is now commonly held that insulin does not increase the rate of entrance of glucose into the erythrocytes of man and mammals. However, the literature on erythrocytes is, or at least has been, highly controversial (see Foshay, 1925; Häusler and Loewi, 1925; Loewi, 1927; Högler, Thomann and Überrack, 1929; Himmerich and Tschernjak, 1936; also many papers cited by them; Wilbrandt, 1947; Guensberg, 1947; Pletscher, von Planta and Hunzinger,

TABLE II

Effect of solutions of glucose and glucose + insulin on the osmotic behavior of Spisula eggs. Measurements of diameters were made after the eggs had been immersed in the solutions for 5 minutes. The values are in microns; they show the average of 10 measurements

	0.9 *M*	0.8 *M*	0.7 *M*
Diameter of eggs in sea water	61.0 ± 2.28	56.8 ± 1.67	56.4 ± 1.07
Diameter of eggs in glucose	56.8 ± 1.78	56.4 ± 1.52	58.9 ± 0.83
Diameter of eggs in glucose + insulin	53.7 ± 1.77	53.5 ± 3.80	54.1 ± 2.87

TABLE III

Effect of glucose and glucose + insulin on the osmotic behavior of Chaetopterus eggs.
Solution A = 4 parts molar glucose solution + 1 part sea water.
Solution B = 4 parts molar glucose solution containing insulin + 1 part sea water

Control eggs in sea water measure 99.4 ± 2.06 microns

Eggs in A (glucose alone) measure		Eggs in B (glucose + insulin) measure	
After 3 minutes	94.0 ± 1.63 microns	After 3 minutes	94.2 ± 1.83 microns
After 136 minutes	95.4 ± 2.50 microns	After 135 minutes	94.0 ± 2.58 microns

1955). Any interpretation of this literature is complicated by the fact that glycolysis and other changes in carbohydrates may well occur in blood cells. Park and Johnson (1955) failed to find any increase in the rate of entrance of glucose and galactose into rat brain cells when insulin was present, but here, too, the results are based on the assumption that under the conditions of the experiment both glucose and galactose remained unaltered when they entered the cells, and this conclusion may not be entirely warranted (compare Sols and Crane, 1954).

If, as is now commonly believed, the favorable effect of insulin on the transport of sugar into cells is due to some sort of an enzyme-controlled reaction, then acceleration of this transport promoting enzyme might well be the cause of the increase in the rate of entrance of sugar. Thus the more rapid transport of sugar into cells in the presence of insulin might merely represent one aspect of the general effect of insulin in accelerating diverse types of enzyme activity. In other words, the more rapid entrance of sugar, instead of being the basic reason for insulin action, might be a result rather than a cause of some underlying change that is responsible for a general increase in enzymic activity. What could such a cause be?

EFFECT OF INSULIN ON COLLOIDAL CHANGES IN PROTOPLASM

When various types of cells are excited by stimuli of one sort or another, calcium is released from the cell cortex, and this calcium then activates a proteolytic enzyme system. The proteolytic enzyme also serves as a clotting enzyme and produces a gelation of protoplasm in the interior of the cell. This gelation involves an oxidation of –SH to S–S groups. Thus the release of calcium can result in an increase in protease activity and also an increase in cellular oxidations. In other words, calcium release is the trigger that starts off a number of enzymic reactions. The evidence on which these statements is based has been presented in considerable detail in recent books (Heilbrunn, 1956, 1958); see also Wilson and Heilbrunn, 1957.

Could it be possible that in one way or another insulin might act in somewhat the same way that stimulating agents do, and what could conceivably be the reason for such an action? This possibility is what intrigued us and induced us to undertake the work that is described below.

If gelation and the reactions underlying gelation in protoplasm constitute the trigger for protoplasmic activity, then presumably protoplasm must have some

method of braking or inhibiting the gelation. In the books just referred to, strong evidence is presented to show that heparin and heparin-like substances can constitute such a brake. Heparin not only can inhibit protoplasmic gelation in much the same way that it inhibits blood clotting, it can also inhibit the action of various types of enzymes. In living cells generally, there seems to be a balance between the factors which tend to induce gelation or clotting and those which tend to prevent it. Heparin (and/or heparin-like substances) is one of the inhibiting factors. If we could imagine a substance which would antagonize or neutralize the effect of heparin and similar substances, then it might well act to accelerate various enzyme systems in the protoplasm. Insulin is such a substance, as we shall now attempt to show.

Sol-gel reactions undoubtedly occur in many, if not all types of protoplasm, but they are especially evident in the ameba. Moreover, in the ameba, a small amount of heparin can be shown to prevent the clotting reaction which normally occurs whenever the cell is torn or broken, that is to say the surface precipitation reaction. We use the giant ameba, *Chaos chaos*, and the heparin we used in our experiments was kindly supplied by the Upjohn Company. If an ameba is immersed in a dilute solution of heparin, say a 0.01% solution, and the ameba is crushed by exerting pressure on the coverslip over the animal, no surface precipitation reaction occurs and the contents of the ameba flow out through the solution. However, if the heparin solution is also made to contain a 0.01% solution of insulin, there is an excellent surface precipitation reaction and the exuding protoplasm forms a distinct membrane about itself.

We performed a series of experiments in which various concentrations of insulin were balanced against various concentrations of heparin. In deciding whether or not a surface precipitation reaction occurs, it is important not to vary too greatly the amount of pressure with which the ameba is broken. For with too great pressure and with too rapid emergence of the interior protoplasm, there is scarcely time for a proper reaction to occur. It is difficult to measure the amount of pressure applied to a coverslip. In order to measure this pressure, one of us (Ashton) devised an apparatus in which a small rectangular piece of glass was attached to a lever which in turn was attached to a DeNouy tensiometer. With this apparatus it is possible to measure the amount of pressure applied before a cell breaks. The measurements are not very exact, but they have the advantage of being objective. For cells which do not vary greatly in volume, as for example sea urchin eggs, the pressure required to break them, as indicated by our apparatus, is reasonably constant. However, for amebae which differ markedly in size, as do our specimens of *Chaos chaos*, the breaking pressure varies more widely, for with the larger amebae there is more resistance to the pressure imposed upon them. Table IV shows what happens when amebae are broken in mixtures of insulin and heparin. The last column indicates whether or not a surface precipitation reaction occurred and whether it was a strong or a weak reaction. The amount of pressure required to break the amebae is also recorded. As was to be expected, this pressure varied widely, but whether the pressure was relatively great or relatively small, the results were always the same. It is clear, therefore, that the effect of heparin in preventing the protoplasmic clotting necessary for the surface precipitation reaction can be completely blocked by the addition of insulin. Moreover, a control test showed

that the amount of zinc present in our solutions had no such effect. It should be noted that a given amount of insulin can neutralize four times as much heparin.

Further evidence of a combination between insulin and heparin is provided by experiments in which it was shown that the metachromatic reaction of heparin with toluidine blue was prevented by solutions of insulin. In these experiments, shown in Table V, relatively large amounts of insulin were necessary to block completely the metachromatic reaction. Here we are dealing with a system in which only insulin and heparin are present, whereas in the earlier experiments the system included not only insulin and heparin, but also the protoplasm of the ameba. Probably the protoplasm, or rather some proteins contained in it, have an affinity for heparin and can unite with it in spite of the presence of insulin. At any rate, this might constitute an explanation of the different types of ratios obtained in the two experiments. Another explanation might be that it may take more insulin to block

TABLE IV

The effect of mixtures of heparin and insulin on the surface precipitation reaction of Chaos chaos

% insulin	% heparin	Ratio insulin/heparin	Pressure in milligrams	Spr
0.01	0.01	1–1	120	strong
		1–1	88	strong
		1–1	92	strong
		1–1	120	strong
		1–1	160	strong
0.01	0.02	1–2	208?	strong
		1–2	140	strong
		1–2	116	strong
		1–2	52	strong
		1–2	96	strong
0.01	0.03	1–3	116	weak
		1–3	124	strong
		1–3	188	weak
		1–3	48	weak
		1–3	116	weak
0.01	0.04	1–4	48	weak
		1–4	168	weak
		1–4	128	weak
		1–4	252?	very weak
		1–4	76	very weak
0.01	0.05	1–5	96	none
		1–5	60	none
		1–5	134	none
		1–5	68	none
2×10^{-7} *M* zinc	0.01		124	none
			125	none
			144	none
			172	none
			145	none

TABLE V

Metachromatic reaction of mixtures of heparin and insulin. One milliliter of a 0.02% insulin solution was mixed with an equal volume of various concentrations of heparin, and the various mixtures were then tested for metachromasia with 6 drops of a 0.01% solution of toluidine blue

% insulin	% heparin	Ratio insulin/heparin	Reaction
0.02	0.02	1	+
0.02	0.01	2	+
0.02	0.0067	3	+
0.02	0.005	4	+
0.02	0.004	5	+
0.02	0.0033	6	+
0.02	0.00286	7	+
0.02	0.0025	8	+
0.02	0.0022	9	+
0.02	0.002	10	+
0.02	0.00182	11	+?
0.02	0.00167	12	+?
0.02	0.00154	13	−
0.02	0.00142	14	−
0.02	0.00134	15	−

the metachromatic reaction of heparin than it does to block its effect on clotting or on enzymic action.

When amebae are stained with toluidine blue, the outer region of the cell gives a beautiful metachromatic color, a color such as that which would be given by heparin or a heparin-like substance. But if amebae are immersed in solutions of insulin for some hours, staining with toluidine blue no longer gives a metachromatic reaction. Such a loss of the metachromatic reaction occurs even in very dilute solutions of insulin. This is shown in Table VI. In interpreting this table, it should be remembered that the amebae were immersed in solutions whose volume was very large in comparison with the volume of the amebae. Actually in the experiments reported in the table, 10 ml. of solution were used and only a few drops of a concentrated suspension of amebae.

Our experiments indicate that insulin can and does combine with heparin. There is some indication in the chemical literature in support of this view. According to Gorter (1954), heparin can combine with various proteins, including insulin. In Gorter's experiment, the insulin was combined with the lipid cephalin

TABLE VI

Metachromatic reaction of Chaos chaos after the amebae were immersed for 16 hours in various concentrations of insulin solution

Concentration of insulin, %	Reaction
0	+
0.000625	+
0.00125	−
0.0025	−
0.005	−

(phosphatidyl-serine), and Gorter believes that as a result of the complex formed between insulin and heparin, the lipid is set free. The reaction between heparin and insulin is strongly influenced by hydrogen ion concentration, a fact which may be of considerable importance in the interpretation of biological phenomena.

Discussion

Although the permeability theory of insulin action has been so widely accepted, as already noted, the fact that insulin increases the rate of passage of sugar into a cell could well be the result of some acceleration of an enzyme responsible for such transport, so that the more rapid entrance of the sugar would really be a result rather than a cause of enzyme action. A somewhat similar idea was expressed many years ago by Staub (1927). But this type of objection is perhaps not too serious. The value of a theory lies in the extent to which it can explain and interpret known facts. Perhaps the most important fact about the action of insulin is that it behaves as an anabolic hormone and produces an increase in the synthesis of carbohydrates, proteins and fats. It could of course be claimed that inasmuch as insulin hastens the entrance of sugar into a cell, this fact in itself might favor the synthesis of proteins, for the energy for such syntheses is now believed to come from the oxidation of carbohydrate. This may well be the correct explanation. However, according to Sinex, MacMullen and Hastings (1952), the addition of glucose tends to prevent the insulin-induced synthesis of protein in the rat diaphragm. And if we consider the long-known facts concerning the effects of insulin, the permeability theory would have some difficulty in offering a complete explanation. For if the primary effect of the hormone is to increase the sugar content of a cell, then logically one should be able to imitate the effects of insulin merely by feeding or injecting excess glucose, for such glucose would also increase the cellular sugar. But administration of excess sugar is hardly a cure for diabetes. Moreover, under conditions in which there is an excess of insulin in the blood, if the sugar permeability theory were correct, from a logical standpoint the very worst possible treatment to offset the harmful effects of the excess would be to feed or inject glucose; for the permeability theory assumes that insulin acts by introducing more glucose into the cells. And yet, as is standard knowledge, the convulsions and other adverse symptoms induced by excess insulin can be cured readily enough by the administration of glucose.

Of course in an animal as complicated as a mammal, there are too many interactions of various organ systems to enable one to reach unassailable conclusions by logic based on the behavior of any one organ system. It is quite possible that insulin and sugar have one effect on muscle and another on the cells of the medulla and spinal cord. Thus it might be postulated that in insulin shock, the nerve cells in the basal part of the brain come to lack glucose and that this lack is responsible for the convulsions and the coma. Then the administration of sugar might quickly restore the nerve cells to their normal state.

On the basis of our theory, if insulin acts primarily by combining with heparin and counteracting its effects, then such an action would immediately accelerate various types of enzymic activity. For, as is well known, heparin is an inhibitor of some proteases. Thus, it inhibits the action of trypsin (Horwitt, 1940), and pepsin (Marini and Levey, 1955). It also inhibits the action of ribonuclease

(Zöllner and Fellig, 1953), and of amylase (Myrbäck and Persson, 1952). Because it is a polyanion, it can have a retarding effect on the activity of various enzymes (Spensley and Rogers, 1954). When injected into the blood stream, it tends to extract lipases from tissues (Iselin and Schuler, 1957), and presumably this would retard cellular lipase activity. And inasmuch as it retards the activity of ribonuclease, it might also tend to prevent the synthesis of ribonucleoproteins; this might be a factor in retarding the formation of proteins with enzymic activity. Finally, because heparin prevents the clotting of protoplasm, and such clotting, as stated previously, acts as a trigger for oxidative reactions, it might also retard the oxidative activity of a cell. Hence by the simple combination with heparin, insulin could exert many of the effects we know it to have. In support of our point of view, it might be noted that according to Bond and Spitzer (1955), much of the hypoglycemic effect of insulin is lost if it is injected into rabbits previously injected with heparin. Bond and Spitzer do not believe that this phenomenon is due to any combination of heparin with insulin, for when they injected a mixture of the two substances, insulin action remained unimpaired. But within blood, and even more within cells, various factors such as pH, ionic strength or even the presence of protein co-factors, might have an influence on any possible combination.

As recent authors are coming to realize (Weissbecker and Hitzelberger, 1953; Riley, Shepherd, West and Stroud, 1955), heparin has many physiological actions in addition to its effect on blood clotting. Thus Riley *et al.* suggest "that the function of heparin may be concerned rather with events in the tissues than with the coagulability of the circulating blood," and a similar statement is also made by Weissbecker and Hitzelberger. One interesting phenomenon is the fact that heparin antagonizes the effects of ACTH and cortisone. As we learn more about the heparin and heparin-like substances that are found in cells, we may gain additional insight into life processes and the action of various drugs on these processes.

Obviously the work we have done represents only a beginning. If the theory we propose is correct, then much more work needs to be done in order to place it on a firm footing. Any theory which attempts to give a complete explanation of insulin activity is faced with many difficulties.

Summary

1. Insulin does not speed the entrance of glucose into the eggs of a sea urchin, a clam and a worm.

2. Dilute solutions of heparin prevent protoplasmic clotting in ameba. This action of heparin is blocked by insulin.

3. Evidence is presented to show that insulin combines with heparin. It blocks the metachromatic reaction that heparin gives with toluidine blue. This can clearly be shown *in vitro*, and it is also indicated by studies on living amebae.

4. Earlier work has shown that heparin acts as an inhibitor of various enzymes, and in general it may be thought of as constituting a brake on many of the chemical activities of a cell. By preventing this inhibiting action, insulin is able to promote the synthesis of various essential constituents of the protoplasm.

5. Also, in view of the fact that protoplasmic clotting involves oxidation and can act as a trigger for oxidative activity, insulin by preventing the anticlotting action of heparin can promote oxidations.

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THE EFFECTS OF CERTAIN NEUROHUMORS AND OF OTHER DRUGS ON THE VENTRICLE AND RADULA PROTRACTOR OF BUSYCON CANALICULATUM AND ON THE VENTRICLE OF STROMBUS GIGAS [1, 2]

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Krijgsman and Divaris (1955) called attention to the need for pharmacological information about the heart of *Busycon canaliculatum*. Such information was gained in the course of investigations of the physiology of *Busycon*, carried out between 1953 and 1956, and is presented here. For the sake of comparison, experiments on the ventricle of *Busycon* were repeated on the ventricle of *Strombus gigas*. Further experiments with the *Strombus* heart are also reported here. The *Busycon* radula protractor (recommended for physiological investigation by Herrick, 1906) was used for a comparison of the effects of the same drugs on non-cardiac muscle.

I wish to thank Professor John H. Welsh for the suggestion which led to this study and for his guidance.

Pharmacology of the ventricle

Methods

The amplitude of heart beat was measured on kymograph records from isolated ventricles, perfused with sea water through the auricle in a manner similar to that described by Welsh and Smith (1949) for larger crustacean hearts. The bath was so arranged that it could be flushed with sea water while the ventricle was washed through the cannula between tests. Drugs in sea water solution were applied by substitution for the perfusion fluid. Experiments on the *Busycon* ventricle were carried out at room temperature of 23° C., and experiments on the *Strombus* ventricle were carried out at room temperature which varied between 20° and 25° C.

Results

Acetylcholine produced a decrease in amplitude of beat in the *Busycon* ventricle at a 10^{-9} molar concentration, with diastolic arrest at 10^{-7} molar (Fig. 1, A). The

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[2] This paper, in slightly different form, comprised part of a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Harvard University.

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[4] Contribution Number 249 from the Bermuda Biological Station. Assisted by a National Science Foundation grant-in-aid through the Bermuda Biological Station.

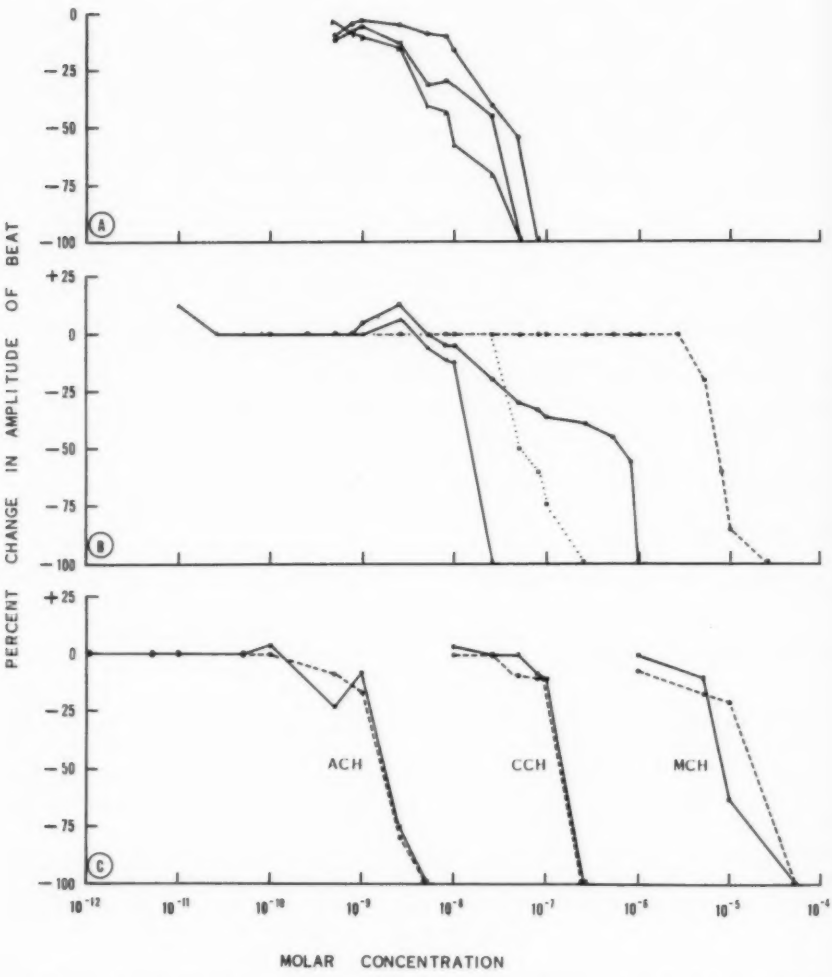

FIGURE 1. A. The effect of acetylcholine on three *Busycon canaliculatum* ventricles. Each point represents the average of the responses of a ventricle to two exposures to the same concentration, once in an ascending series, and once in a descending series. B. The effect of acetylcholine (solid lines), carbamylcholine (dotted line), and acetyl-beta-methylcholine (broken line), on the *Strombus gigas* ventricle. C. The effect of eserine on the concentration-action curve of the *Strombus gigas* ventricle for acetylcholine (ACH), carbamylcholine (CCH), and acetyl-beta-methylcholine (MCH). In each case the solid line represents the effect of the ACH or ACH analogue in the perfusion sea water of an uneserinized heart. The broken line represents the effect of the ACH or ACH analogue applied in perfusion fluid, consisting of a 10^{-5} molar sea water solution of eserine, to a ventricle previously soaked for an hour in 10^{-5} molar eserine.

threshold to acetylcholine was found to be approximately the same for *Strombus,* but the response to a concentration just above threshold was an increase in amplitude, with a decrease in amplitude elicited by concentration ten times threshold (Fig. 1, B). In both *Strombus* and *Busycon,* acetylcholine improved irregular beating at concentrations ten times less than the level of the threshold for an effect on amplitude.

Although carbamylcholine in low concentrations failed to produce the increase in amplitude of the *Strombus* ventricle beat that was seen with acetylcholine, it produced decrease in amplitude in the neighborhood of 5×10^{-8} molar concentration (Fig. 1, B).

Acetyl-beta-methylcholine has an acetylcholine-like effect on the *Strombus* ventricle but with a threshold concentration one thousand times greater (Fig. 1, B).

Eserine failed to potentiate the action of acetylcholine, carbamylcholine, or acetyl-beta-methylcholine on the *Strombus* ventricle. It did regularly abolish the excitatory effect of low concentrations of acetylcholine (Fig. 1, C).

Both adrenalin and noradrenalin proved to have a positive tonotropic effect on the *Busycon* ventricle but the effective concentrations were not in the extremely dilute range at which acetylcholine became effective. At a 10^{-5} molar concentration either neurohumor increased the amplitude of beat about fifty per cent, but the amplitude was increased nearly one hundred per cent when a 10^{-5} molar concentration was obtained as the sum of the molarities of adrenalin and noradrenalin added simultaneously (Fig. 2, A).

5-Hydroxytryptamine was found to have an action on the *Busycon* heart similar to that of adrenalin but with a threshold in the vicinity of 10^{-9} molar, and is thus active in dilutions comparable to acetylcholine dilutions. The *Busycon* ventricle is a thousand times less sensitive to tryptamine than to 5-hydroxytryptamine (Fig. 2, C). Adrenalin or noradrenalin concentrations fifty times greater than threshold concentration for a particular heart irreversibly stop the heart, but 5-hydroxytryptamine at 10^{-2} molar, ten million times the threshold concentration, does not even produce systolic arrest.

In contrast to the synergistic effect of simultaneous addition of adrenalin and noradrenalin to the perfusion fluid, when adrenalin and tryptamine are added to the perfusion fluid simultaneously the effect is not significantly greater than if the same molar concentration were made up of one drug (Fig. 2, B).

5-Hydroxytryptamine acts on the *Strombus* ventricle over a wide range of concentrations with a threshold at 10^{-10} molar (Fig. 3, A).

The antagonism between the negative tonotropic effect of acetylcholine and its analogs, and the positive tonotropic effect of 5-hydroxytryptamine, on the *Strombus* ventricle, is plotted in Figure 3, B, in terms of the reduction by acetylcholine, carbamylcholine, or acetyl-beta-methylcholine of the amplitude maintained by 10^{-7} molar 5-hydroxytryptamine. Acetylcholine acts on the 5-hydroxytryptamine excited ventricle much as on the normal ventricle, but carbamylcholine, which is less effective than acetylcholine in depressing the spontaneous heart beat, is almost as effective an antagonist of 5-hydroxytryptamine as is acetylcholine. Gramine is another antagonist of the action of 5-hydroxptryptamine on the *Strombus* ventricle, and will completely block the action of 10^{-7} molar 5-hydroxytryptamine at a 5×10^{-5} molar gramine concentration.

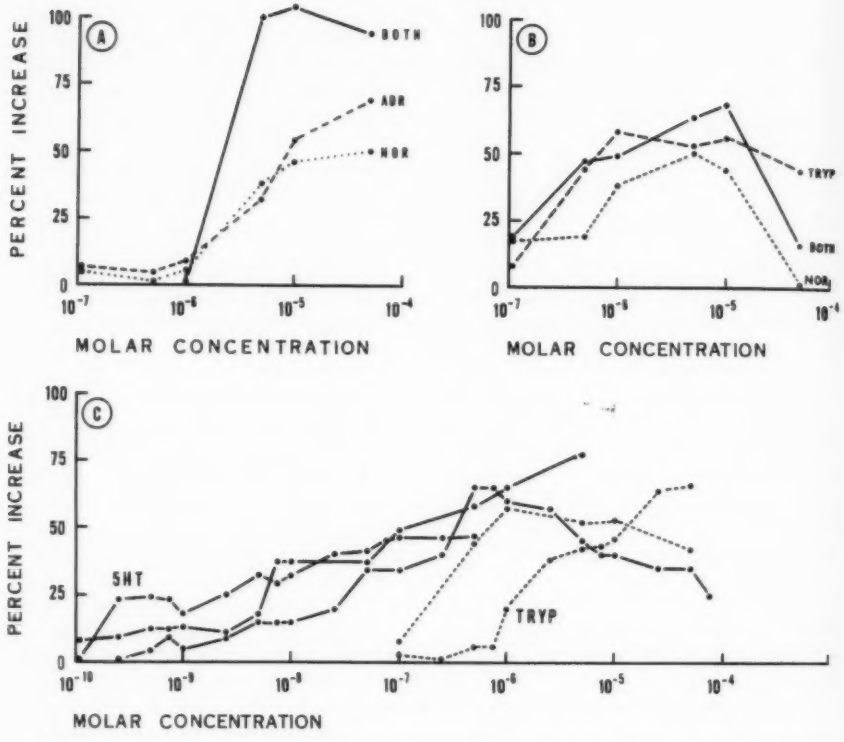

FIGURE 2. A. The effects of adrenalin, of noradrenalin, and of the same molar concentrations made up with equal amounts of the two amines, on the amplitude of beat of the isolated *Busycon canaliculatum* ventricle. Each curve ends at the concentration at which the ventricle stopped in systole. B. A similar comparison of the effects of tryptamine, of noradrenalin, and of both simultaneously on the *Busycon canaliculatum* ventricle. C. The effects of tryptamine and 5-hydroxytryptamine on the amplitude of beat of the isolated *Busycon canaliculatum* ventricle.

DISCUSSION

The pharmacological relations of the *Busycon canaliculatum* ventricle resemble those of other gastropod hearts. Acetylcholine has been shown to depress the beat of the hearts of the gastropods *Buccinum undatum* and *Cyprina islandica* (Welsh, 1956), *Dolabella auricula* (Ebara, 1955), *Cochlitoma zebra* (Divaris and Krijgsman, 1954), *Helix pomatia* (Jullien and Ripplinger, 1950), and *Murex trunculus* (Jullien and Morin, 1931).

Among the hearts listed above, *Murex trunculus* has been reported by Morin and Jullien (1930) to have a small group of nerve cell bodies near the location where Carlson (1905) reports a ganglion in *Busycon*. However, Divaris and Krijgsman (1954) not only found no nervous elements in the white spot at the *Cochlitoma zebra* ventriculo-aortic junction, but also demonstrated the existence of

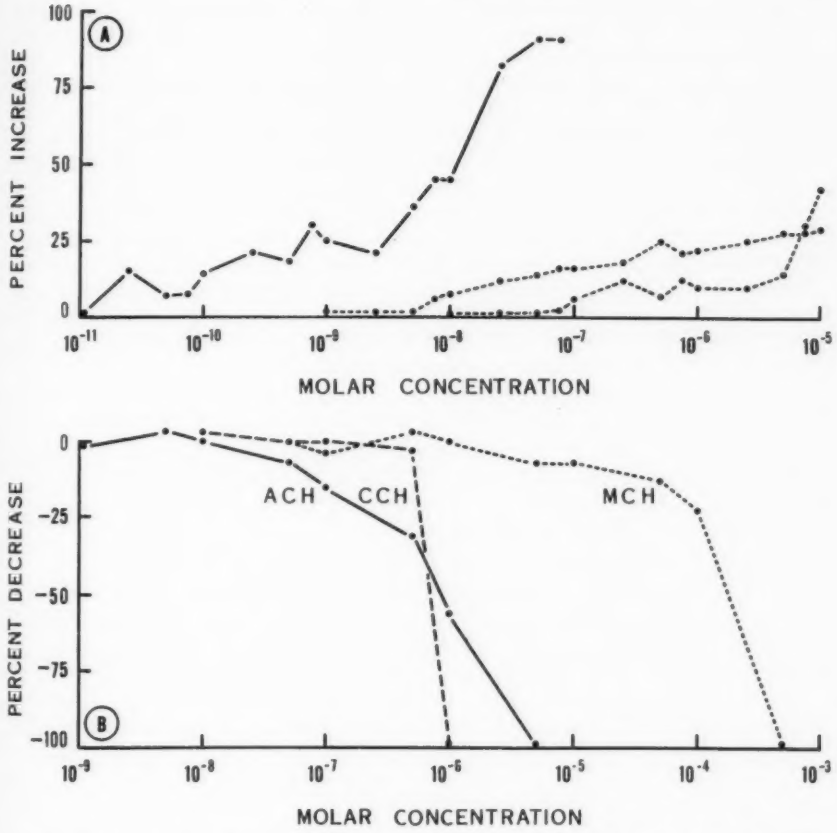

FIGURE 3. A. The effect of 5-hydroxytryptamine on the amplitude of beat of the isolated ventricles of *Strombus gigas* (solid line) and *Aplysia protea* (broken line). B. The effects of acetylcholine, carbamylcholine, and acetylbetamethyl choline, on the amplitude of beat, of the *Strombus gigas* ventricle, which is maintained by perfusion with 10^{-7} molar 5-hydroxytryptamine.

myogenic pacemakers. A myogenic origin for the beat of the heart of *Murex trunculus* was indicated when Cardot, Jullien and Morin (1929) showed that isolated fragments would beat in sea water. Thus, the *Busycon* ventricle reacts to acetylcholine like the hearts of other gastropods which have been demonstrated to have myogenic pacemakers.

The extremely low concentrations at which acetylcholine and 5-hydroxytryptamine are effective on the *Busycon* ventricle are in accordance with Welsh's (1957) finding that they act as neurohumors in *Venus mercenaria*. That 5-hydroxytryptamine is effective in lower concentration than adrenalin or noradrenalin, with an equally rapid onset of action, suggests that 5-hydroxytryptamine might

be closer in structure to the natural cardio-regulatory neurohumor of *Busycon* than are the mammalian neurohumors. 5-Hydroxytryptamine has, in fact, been found in the pooled ganglia of *Busycon* by Welsh (1954).

The response of the *Busycon canaliculatum* ventricle to adrenalin and noradrenalin is evidence that it is more similar pharmacologically to the hearts of the molluscs *Eledone cirrosa* and *Anodonta* (Fänge and Ostlund, 1954), which respond by an increase in amplitude of beat, than to that of *Aplysia dactylomela*, which is insensitive to the two neurohumors, as reported by von Euler, Chavez and Teodosio (1952). That the isolated *Busycon* heart beats so well is also a contrast to the *Aplysia dactylomela* heart which will beat spontaneously only if adjacent ganglia are isolated with it (von Euler, Chavez, and Teodosio, 1952). However, I have found the isolated ventricle of *Aplysia* to beat spontaneously, although not well. Its beat may be sustained by ergonovine or by 5-hydroxytryptamine, but its threshold to 5-hydroxtryptamine is between 10^{-8} and 10^{-7}, which is considerably higher than the thresholds of the *Busycon* and *Strombus* ventricles (Fig. 3, A).

Pharmacology of the leached ventricle

Following Burn's theory (1950) of the relation of local hormones to cardiac automatism, it might be expected that a denervated ventricle, which had been deprived, by leaching, of previously synthesized neurohumors, would respond by contraction to either acetylcholine or 5-hydroxytryptamine.

Methods

In order to ascertain the upper limit to the time an isolated *Busycon canaliculatum* ventricle may remain viable and useful for bioassay, six isolated entire hearts were set aside in sea water at 9° C. for periods ranging from one to six weeks. At intervals, a ventricle was removed to room temperature, allowed five hours for adjustment, and then perfused. It would seem probable that after a week the cut distal portions of the cardioregulatory nerves (from the visceral ganglion; Carlson, 1905) would have degenerated, so that the leached ventricle might react to pharmacological agents primarily as a muscle preparation.

Results

Of two hearts kept at 9° C. for one week, both survived and both beat normally when perfused. That is, after one-half hour one was beating at 18 systoles per minute, the other at 21 and both were emptying completely at each systole. Each continued at its original rate for 5 hours, at the end of which time one showed a threshold response to 5×10^{-9} *M* 5-hydroxytryptamine and 10^{-9} *M* acetylcholine, and the other a threshold response to 10^{-9} *M* acetylcholine and to 10^{-9} *M* 5-hydroxytryptamine.

Of the two hearts set aside for three weeks, only one survived. The other, after one-half hour of perfusion, was beating regularly at a beat of 22 systoles per minute. After three hours it had slowed down to 12 systoles per minute but was still beating regularly. Now, however, the ventricle was no longer emptying completely and relaxed to three times its previous volume at diastole. By increasing the pressure of perfusion the rate was increased to 18 systoles per minute and, while the ventricle

retained the full relaxation at diastole, it returned to complete emptying at systole. After eight hours of perfusion, the rate was down to nine systoles per minute but was restored to eighteen by an increase in pressure. After ten hours of perfusion the rate had dropped again to ten systoles per minute and a further increase in pressure was required to raise it to sixteen. After this ventricle, which had been set aside for three weeks in sea water at 9° C., had maintained an uninterrupted rhythm for fourteen hours and sixteen minutes its threshold for 5HT was found to be in the neighborhood of a 5×10^{-9} molar concentration and its threshold for acetylcholine was 10^{-10} molar.

Of the two hearts set aside for six weeks at 9° C., one survived. When set up in a heart bath and perfused with sea water the ventricle showed no sign of spontaneous activity, but it did react to 10^{-7} molar 5HT by beating at the rate of 18 systoles per minute as long as it was subjected to 5HT.

Subsequently, four hearts were taken which had survived four weeks at 11° C. but which did not beat spontaneously when perfused. Each was subjected to concentrations of acetylcholine and of 5-hydroxytryptamine from 10^{-12} to 10^{-2} molar at half-molar intervals. No concentration of acetylcholine provoked beating. All four hearts beat, when perfused with 10^{-7} molar 5-hydroxytryptamine, at a normal rate but at an amplitude much less than that which had been elicited by 5-hydroxytryptamine after soaking at 9° C. for six weeks.

Discussion

The failure of acetylcholine to restore automatism is in accord with the similar findings of Jensen (1957) with several lamellibranch hearts.

Pharmacology of the radula protractor

Methods

The radula protractor was isolated intact, attached to a bit of radula sac at one end, and to a fragment of odontophore at the other, and set up in a sea water bath at 19° to 21° C. Drugs in sea water solution were added to the bath to produce the desired molar concentration. Ejection from a syringe assured thorough mixing. Air was bubbled through the bath from a capillary tube entering at the bottom.

Results

Spontaneous contractions do not occur in a *Busycon canaliculatum* radula protractor isolated and maintained under slight tension in a constant temperature sea water bath. This makes it a more favorable preparation for the study of induced contractions than the more commonly used snail retractor pharyngis, where rhythmicity obscures the effect of stimulation (Masai, 1951).

Acetylcholine at 10^{-5} molar concentration will induce a contraction which reaches full amplitude immediately. When the acetylcholine is washed off, the muscle relaxes immediately. If the acetylcholine solution is left in contact with the muscle, the contraction declines slightly for five or ten minutes, and then the rate of relaxation accelerates, and by forty-five minutes after adding acetylcholine

the muscle is relaxed again. The bathing solution has then lost its ability to contract a fresh muscle. Normal responsiveness of the muscle is restored by ten minutes of washing with aerated sea water.

When 10^{-5} *M* acetylcholine is followed in three minutes by sufficient tryptamine to produce a concentration in the bath of 10^{-3} *M*, relaxation is not an immediate fall in tension such as is seen when acetylcholine is washed off, but is gradual at first and then develops into rhythmic pulsation with a slowly declining base line (Fig. 4, A). The rhythmicity may be as regular in rate and amplitude as a heartbeat and ceases abruptly with the relaxation that follows when the mixture of the two drugs is washed off. In each of the rhythmic contractions the radula protractor shortened to about one-third of its resting length. Figure 4, B, shows the similar response when the muscle is subjected first to 10^{-5} *M* acetylcholine and then in two minutes to the combination of 10^{-5}*M* acetylcholine and 10^{-3} *M* 5-hydroxy-

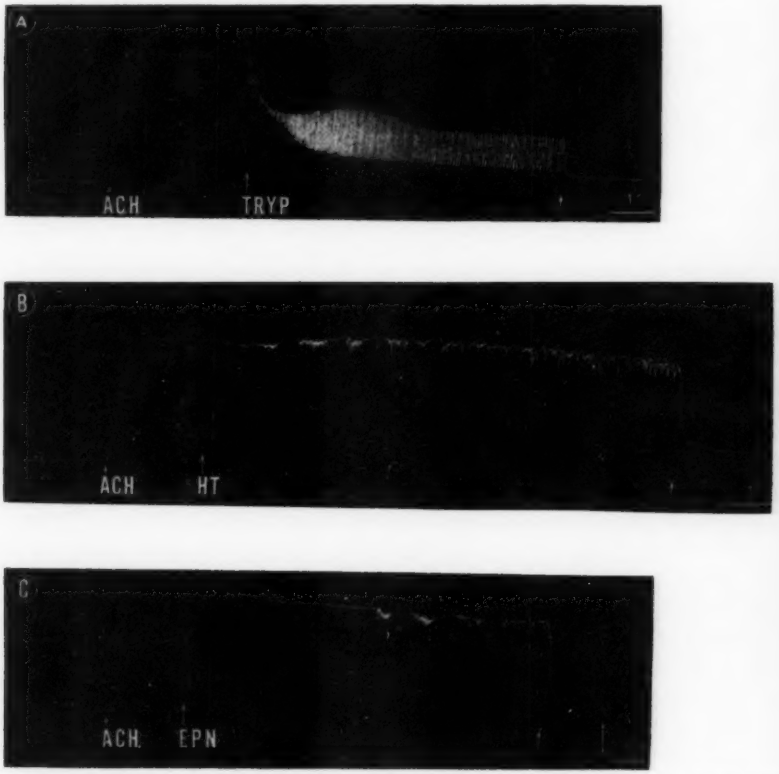

FIGURE 4. Responses of the *Busycon canaliculatum* radula protractor to 10^{-5} molar acetylcholine (ACH) followed by 10^{-3} molar tryptamine (TRYP), 5-hydroxytryptamine (5-HT), or adrenalin (EPN).

tryptamine. Figure 4, C, shows the response obtained when the radula protractor is subjected first to 10^{-5} *M* acetylcholine and then in two minutes to 10^{-3} *M* adrenalin, also. These combinations were found to be optimum for regularity and amplitude of "beat," and it may be seen that tryptamine was the most effective of the three amines.

Figure 5, A, shows that in the radula retractor, tryptamine following acetylcholine produces a similar rhythmic decline of tension. The same is true for the odontophore retractor (Fig. 5, B). It may be seen that in Figure 5, A, the first effect of tryptamine was a slight decline in tension while in Figure 5, B, it was a slight increase. The first effect seems to vary randomly for all three radula apparatus muscles, but is always followed by the rhythmic relaxation.

In Figure 5, A, at T_1, 10^{-5} *M* tryptamine was added in the absence of prior stimulation by acetylcholine, and at T_2, the concentration was brought to 10^{-4} *M* tryptamine. Neither contraction nor relaxation was elicited, yet at the second T_2 the same concentration (following acetylcholine) produced relaxation. At the temperature of these experiments, 19-21° C., no concentration of tryptamine, 5-hydroxytryptamine, or adrenalin relaxed a radula apparatus muscle that had not been previously excited to contract. Later it was found that at 27° C. 5-hydroxytryptamine would cause a previously unstimulated muscle to contract slowly and irregularly, but never to relax.

A radula protractor in 10^{-3} *M* 5-hydroxytryptamine develops a sensitivity to stretching not shown in the muscle simply isolated in sea water. It responds to a sharp tug by a quick contraction followed by slower relaxation.

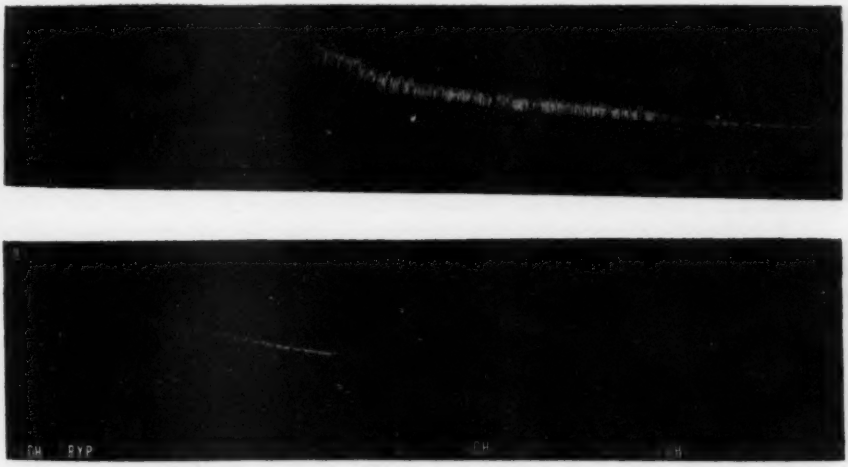

FIGURE 5. *Busycon canaliculatum.* A. Radula retractor: $T_1 = 10^{-5}$ molar tryptamine, $T_2 = 10^{-4}$ molar tryptamine, $A_1 = 10^{-5}$ molar acetylcholine. B. Odontophore retractor: ACH = 10^{-4} molar acetylcholine, TRYP = 10^{-3} molar tryptamine, M = 10^{-5} molar Mytolon for a half hour. A plain arrow indicates that the muscle was washed with sea water with the drum moving, and a double-ended arrow indicates that the muscle was washed with sea water for an hour with the drum stopped.

Acetylcholine contraction of the radula protractor may be blocked both by an agent active at motor end plates, d-tubocurarine, and by the most effective antagonist of acetylcholine on the *Venus mercenaria* heart (Luduena and Brown, 1952), Mytolon. When 10^{-3} molar d-tubocurarine is applied for half an hour the contraction elicited by 10^{-5} molar acetylcholine is blocked but not that due to 0.5% KH_2PO_4. Similarly, 1:10,000 Mytolon applied for a half hour greatly reduces the contraction elicited by 10^{-5} molar acetylcholine but has no effect on the contraction following 0.5% KH_2PO_4.

Acetylcholine contraction of the radula protractor is potentiated by eserine. When the graded responses of the same muscle to an increasing series of acetylcholine concentrations, before and after soaking for an hour in 1:10,000 eserine, are compared, it is found that the response at each concentration is augmented although the threshold to acetylcholine is not altered. Prior soaking in eserine has the same effect on the rhythmicity obtained with acetylcholine and tryptamine as has increasing the concentration of acetylcholine used.

Lysergic acid diethylamide is antagonistic toward the production of a "beat" by the combined action of acetylcholine and tryptamine, but does not itself cause contraction or relaxation at concentrations from 10^{-5} molar to 10^{-10} molar.

Discussion

The rhythmic "beat" of the radula protractor suggests a model of the heart beat. Acetylcholine and 5-hydroxytryptamine both occur as natural neurohumors, both will regulate the *Busycon* heart, and together they induce a rhythmicity in the *Busycon* radula protractor comparable to the automatic rhythmicity of the heart. Tryptamine, however, is more effective than 5-hydroxytryptamine in inducing rhythmicity, whereas 5-hydroxytryptamine is more effective on the heart. Heart strips, when cut to dimensions similar to the radula protractor and set up on the same apparatus, respond to 5-hydroxytryptamine with rhythmic contractions, which are opposed by acetylcholine.

It is tempting to speculate that the radula protractor "beat" might originate in the presence at the cell surface of the opposing neurohumors in the right proportions for alternate action. Welsh and Slocombe (1952) suggest that released acetylcholine depresses the *Venus mercenaria* heart by changing the membrane polarization of muscle fibers and thus interfering with normal contraction and the normal spread of excitation. The effects of acetylcholine and 5-hydroxytryptamine on the surface membrane polarity of a non-cardiac molluscan smooth muscle have been investigated by Twarog (1954). She found that acetylcholine depolarized the *Mytilus edulis* anterior byssus retractor and initiated contraction. 5-Hydroxytryptamine caused immediate relaxation but produced no change in membrane polarization. Furthermore, when the acetylcholine was washed off, the muscle immediately repolarized, but the contraction persisted. (It may be recalled that when acetylcholine was washed off the radula protractor, the muscle relaxed immediately). Twarog suggests that it is probable that the depolarization induced by acetylcholine is directly related to the ensuing contraction. The failure of 5-hydroxytryptamine to produce membrane changes while relaxing the muscle could be attributed to a direct action on the contractile element.

A possible explanation for the rhythmicity induced in the radula protractor by

acetylcholine and tryptamine could be based on Twarog's byssus retractor results. It could be supposed that the acetylcholine in the bath kept the muscle cells depolarized, which would lead to contraction. The tryptamine also in the bath would relax the contractile elements and a second contraction would then occur in response to the surface depolarization.

One alternative hypothesis would be that acetylcholine depolarized the muscle fiber surface membrane and that tryptamine then repolarized it. If it is supposed that contraction follows depolarization and relaxation follows repolarization, the "beat" might be explained. That the applied acetylcholine and tryptamine act on muscle rather than on nerve may be indicated by the persistence of susceptibility to induced "beating" in isolated radula protractors stored for a week.

Summary

1. The hearts of *Busycon canaliculatum* and *Strombus gigas* were found to respond to applied neurohumors as do the myogenic hearts of other gastropods. Acetylcholine was cardio-inhibitory, and 5-hydroxytryptamine was cardio-accelerator, in concentrations low enough to suggest that they might be the normal regulatory neurohumors.

2. The *Busycon canaliculatum* radula protractor was contracted by acetylcholine, and could then be relaxed rhythmically by 5-hydroxytryptamine, tryptamine, and adrenalin, all of which raise the tonus of the ventricle.

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OXYGEN UTILIZATION IN THE SYMBIOSIS OF EMBRYOS OF THE SALAMANDER, AMBYSTOMA MACULATUM AND THE ALGA, OOPHILA AMBLYSTOMATIS

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Unicellular algae occur as symbionts in a great number of aquatic invertebrates, in protozoans, sponges, coelenterates, turbellarians, bryozoans, rotifers, gastropods, lamellibranchs, annelids, and ascidians (Yonge, 1944). An association of unicellular algae and vertebrates has been known only since 1888 when Orr reported a spherical green alga in the egg envelopes of *Ambystoma maculatum* (Shaw). The symbiotic relationship between these salamander eggs and algae was established by Gilbert (1942, 1944) who performed several experiments and made several interesting observations on egg masses of *A. maculatum*.

Masses of these salamander eggs inhabited by the unicellular alga *Oophila amblystomatis* (Lambert) [1] were demonstrated to have lower mortality, an earlier hatching time, and a faster growth rate than algae-free masses: the newly-hatched larvae averaged 1.3 mm. more in length and were more than two Harrison stages in advance of larvae from algae-free egg masses of the same age. Gilbert demonstrated that the results were due to the presence of the algae and not to the presence of light. It was also shown that the algae grow more vigorously in the presence of the embryo than in its absence. Other organisms in addition to the alga inhabit the egg envelopes. These include a protozoan (*Haptophyra* sp.) which feeds on the algae, other protozoa, nematodes, rotifers, acanthocephalans, diatoms (usually on the surface of the egg masses), and even an occasional annelid.

A more rapid growth rate and better viability of embryos from algae-inhabited complexes, as reported by Gilbert, were confirmed during the course of this investigation. These observations suggest that the symbiotic relationship may consist of the utilization by the embryo of the O_2 produced in photosynthesis by the algae and the utilization by the algae of CO_2 produced by the embryo.

Eggs of *Ambystoma maculatum* are often laid in small, temporary, woodland ponds usually containing decaying leaves and other detritus. Such a pond often has a low O_2 content, and any method which would increase the O_2 supply to the embryo might aid in its development. The following experiments were designed to determine if the algae inhabiting the egg membranes produced O_2 in a sufficient quantity to be of value to the developing embryo.

Materials and Methods

Eggs of *A. maculatum* in the early stages of development (Harrison Stages 9–13) were collected from a small, detritus-filled, temporary pond in Duke Forest

[1] See Gilbert (1942) and Smith (1950) for description of the alga and discussions of correct nomenclature.

near Durham, North Carolina, on February 26 and 27, 1958. Several days of cold rains and snow preceded the time of collection. The temperature of the water was between 5° and 6° C. at the time the eggs were taken. Algal growth was not evident in these early stages.

The egg masses were separated into approximately equal halves. One half of each mass was then allowed to develop in the light in pond water collected with the eggs; thick algal growths developed in these eggs. The other half was removed from the pond water, placed in spring water, and kept in the dark; no algal growths were visible in these eggs. Egg masses utilized in preliminary experiments were kept at 6° C., but all other eggs used were kept in a constant temperature cabinet at 20 ± 1° C.

All oxygen consumption measurements were made in a Warburg respirometer at 25° C. Slightly different amounts of water were added to the flasks to equalize the volume of their contents. A period of two hours was allowed for temperature equilibration before O_2 consumption was measured. Flasks were not shaken in the experiments reported here.

Light was excluded from the flasks in some of the experiments by wrapping aluminum foil around the flask and manometer stem or by cutting off all lights in the laboratory during hours of darkness. All other determinations were made with approximately 150 foot candles of light at the level of the flasks, measured with a Weston Model 603 Illumination Meter.

Embryos were separated from their surrounding envelopes by placing them in a measuring spoon slightly smaller than the eggs and pressing them gently against the side of a finger bowl, rupturing the membranes and allowing the embryo to drop out when the spoon contents were submerged in the water of the finger bowl. Six to nine eggs, embryos, or envelopes were then placed in each experimental flask.

All measurements in each category of experiments were made in duplicate, reduced to standard temperature and pressure, and expressed as microliters (μl) per hour per embryo, envelope, or a complex of both embryo and envelope. All determinations were made over a period of at least ten hours.

Although the experiments reported here involve, primarily, the algae and embryo, many other inhabitants are present in the egg envelopes. For this reason we refer to the "complex," meaning the entire egg envelopes together with their contents—embryo, algae, protozoa, bacteria, etc.

Availability of materials in the proper stages of development sometimes limited categories of experiments which could be performed. Oxygen consumption determinations were made on the following categories of experimental materials:

I. Algae-free complexes
   1. Stages 12–13
   2. Stages 32–34
II. Algae-inhabited complexes
   1. Stages 12–13
   2. Stages 32–34
      a. Light
      b. Dark

III. Isolated embryos
  1. From algae-free, stages 39–40
  2. From algae-inhabited
    a. Stages 12–13
    b. Stages 39–40
IV. Isolated envelopes
  1. Algae-free, stages 39–40
  2. Algae-inhabited, stages 39–40

Results

Oxygen consumption was linear over the entire periods of observation as seen in Figure 1, where each point represents the average of duplicate flasks in five of the experiments. Little variation was obtained among flasks containing similar experimental material.

Mean rates of O_2 consumption for each category of experiments are shown in Figure 2. Differences in rate of oxygen consumption at different stages must be considered in interpreting these results. According to Hopkins and Handford (1943), the rate of oxygen consumption begins with a low value and rises gradually to stages immediately preceding hatching (Stages 37–38). At this time the rate increases rapidly to a maximum and then falls off during the last few stages of development.

The O_2 consumption of the algae-inhabited complex (Stages 12–13) averaged 1.41 μl and for the complex without algae, 0.97 μl. This represents an increase of 45.4% when algae are present. In later stages (32–34) of development the values were 1.91 μl for the complex with algae and 1.11 μl for the algae-free complex, an increase of 74.8% when algae are present. Thus, the difference between algae-free and algae-inhabited complexes is in the direction of greater consumption by the latter and increases with stages in development of the embryo.

Oxygen consumption of algae-inhabited complexes (Stages 32-34) determined under conditions of darkness averaged 2.50 μl, an increase of 0.56 μl (28.9%) over that of similar complexes in the light. This latter value can be taken to approximate the respiration of the algae in the dark and may be assumed to represent the net amount of O_2 evolved during photosynthesis of the algae, provided the assumption is correct that the respiration of the algae is balanced by O_2 production during photosynthesis. As pointed out below, if the algae are facultative heterotrophs, this may be an incorrect assumption.

Algae-free envelopes consumed 0.84 μl, while envelopes with algae present consumed only 0.41 μl, a difference of 104%. The consumption by envelopes can be accounted for by the respiration of the bacteria, protozoa, etc., living within the envelopes. The difference in O_2 consumption evidently is due to O_2 production during photosynthesis of the algae. This net reduction of O_2 consumption in the envelopes may be of value to the embryo by decreasing the removal of O_2 from the immediate environment. However, there is a net consumption of O_2 by all envelopes, indicating that there is no surplus of photosynthetically produced O_2 available to the embryo. Therefore, a higher O_2 tension could not account for the faster development and greater respiratory rates of embryos associated with algae.

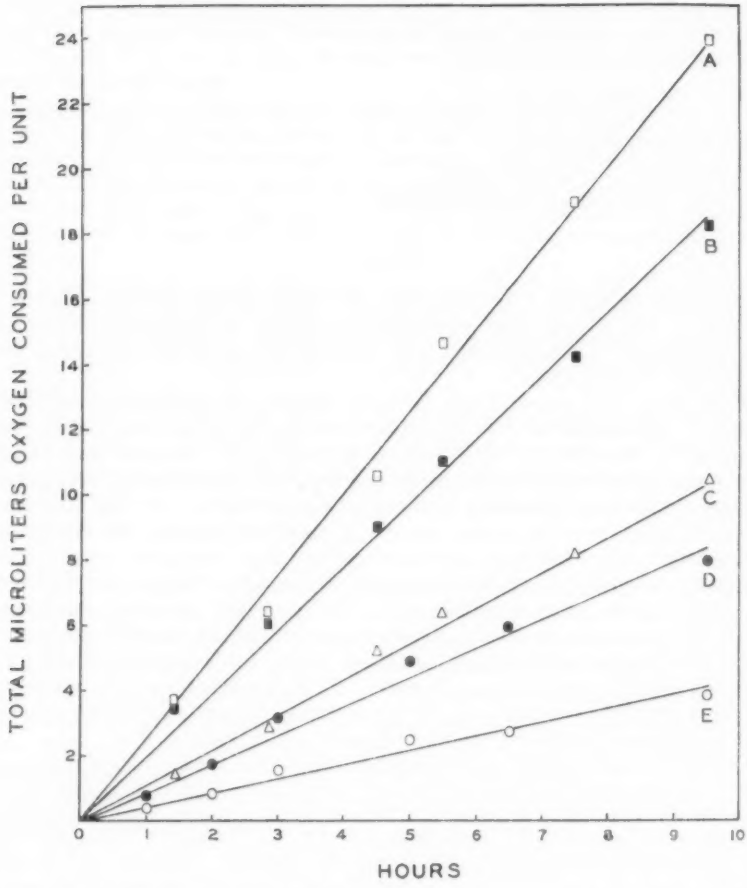

FIGURE 1. Oxygen consumption in five categories of experiments. A, complex with algae in the dark. B, complex with algae in the light. C, complex without algae. D, envelopes without algae. E, envelopes with algae. Embryos in A, B, and C were in stages 32–34.

The difference between O_2 consumption of the isolated envelopes with and without algae in the light was 0.43 μl. This value should approximate the amount of O_2 produced by the algae. If the symbiotic relationship of algae and embryo were only one of respiratory gas exchange, then the consumption of a complex without algae should be greater than a complex with algae, and the subtraction of the amount of O_2 supplied by the algae from the O_2 consumption of an algae-free complex should give a value approximately equal to that of an algae-inhabited complex. The data presented here show that this is not the case. Indeed, such

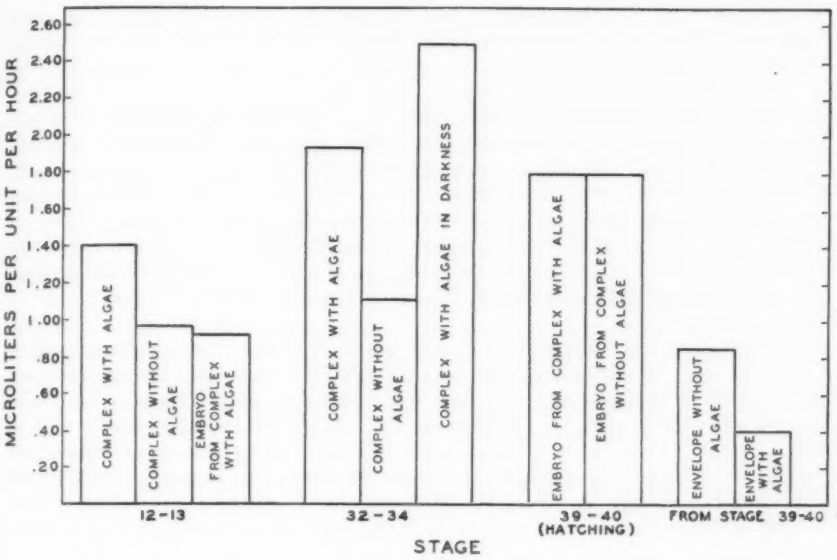

FIGURE 2. Mean rates of oxygen consumption for each category of experiments.

a subtraction only serves to *increase* the difference in respiration rates of algae-free and algae-inhabited complexes.

Isolated embryos of Stages 12–13 consumed an average of 0.94 μl, practically identical with that of intact complexes without algae, containing embryos in the same stages of development. This suggests that the greater consumption of a complex with algae is due primarily to respiration of the embryos themselves, rather than to inhabitants of the envelopes.

Embryos alone in Stages 39–40 consumed the same amount of O_2, whether from algae-free or from algae-inhabited envelopes. At first glance this would seem to contradict the results of other experiments. The fact that these embryos were in the hatching stage probably lends no support to conclusions about their metabolic rates. Developmental changes during this period are rapid, and activity of individual embryos varies widely. Hopkins and Handford (1943) found that the O_2 consumption of *Ambystoma tigrinum* was consistently higher than that of *A. punctatum* during stages of development up to 41–42. At this stage the variations in results were wide, and species differences were obscured.

DISCUSSION

If photosynthesis by the algae resulted in a supply of O_2 for the embryo, it is reasonable to assume, given equal requirements by the embryos, that the net O_2 consumption of algae-inhabited egg masses in the light would be less than the consumption of algae-free complexes. However, the opposite results were obtained.

The results given above indicate that embryos with envelopes containing algae respire at a greater rate than embryos in the same stage of development without the benefit of algae. Furthermore, this increase in O_2 demand is not equaled by the smaller amounts of O_2 evolved by the algae during photosynthesis. These results lead to the conclusion that the symbiotic relationship of embryos and algae is not one of respiratory gas exchange alone, and imply some other factor—possibly a growth-stimulating substance, which would account for higher respiratory rates, faster growth, better survival, and larger size at hatching. The fact that a complex with algae consumes more O_2 (and linearly) in the dark than a similar complex in the light as compared with an algae-free complex, indicates that this growth factor is probably supplied in equal amounts during photosynthesis and in darkness, since the rate of consumption continues at the same high level throughout the ten hours of measurements.

The isolation and identification of such a growth factor might require the use of extracts from the algae grown in pure culture. The fact that the alga involved inhabits only *Ambystoma* egg envelopes (with one exception, where it was found in *Rana sylvatica* envelopes) suggests that the determination of a proper culture medium would not be easily accomplished. If such culture media were developed, the different components of algal extracts added to eggs without algae might lead to the proper identification of the substances involved. The presence of numerous bacteria within the envelopes indicates that the factor involved is probably not an antibiotic.

Several problems are suggested by the presence and better growth of the algae associated with embryos. It is likely that the algae obtain organic nitrogenous substances from the embryo. The observation by Gilbert (1942) that the alga is thickest in the inner envelope and is especially numerous around the proctodaeum of the embryo suggests that nitrogenous wastes or bicarbonates are being utilized by the algae. Experiments by Gregg and Ballentine (1946) indicate the presence of CO_2 as bicarbonate in the blastocoel and archenteron fluid of amphibian embryos. If the alga is a facultative heterotroph, then it is inaccurate to assume that its photosynthesis balances or exceeds its respiration. In addition, the alga is unknown from outside egg masses, and the process used to migrate into and through the gelatinous egg coverings is also unknown.

Buchsbaum (1937) has shown that a metabolic mutualism occurs when a culture of the green alga *Chlorella* is combined with embryonic chick tissue cells, where both were favored over algae alone or chick tissues alone. He found that tissues without algae grew better in the dark than those with algae and concluded that the results were "best explained on the basis of a photosynthetic mutualism." These conclusions were based on measurements of growth alone and not on differences in respiratory rates.

Buchsbaum's data suggest that the mutual benefits derived from an association of *Chlorella* and chick tissue cells are due to gas exchanges involved in photosynthesis, or if some other factors are responsible, they are produced only during photosynthesis. Our results indicate the opposite—that photosynthesis alone cannot account for all of the increased respiration and growth of the embryos, but that other factors are involved in the facultative mutualism of *Ambystoma* embryos and the alga *Oophila*.

We wish to express our appreciation to Dr. H. J. Humm, Dr. I. E. Gray, and Dr. J. R. Gregg for helpful suggestions and critical reading of the manuscript.

Summary

1. Oxygen consumption measurements were made on embryos of the salamander, *Ambystoma maculatum*, which develop within a gelatinous envelope usually inhabited by a unicellular green alga, *Oophila amblystomatis*, and numerous protozoa and bacteria.

2. A comparison of embryos associated with the alga, and others of the same developmental stage but lacking visible signs of the alga, reveal that the former respire at a greater rate in both early and later stages.

3. In darkness the oxygen consumption of the algae-containing complexes is greater than in the light, suggesting that photosynthesis by the algae answers a portion of the oxygen demand. This is also indicated by the fact that isolated green envelopes consume only half as much oxygen as isolated colorless envelopes. Net consumption by the isolated green envelopes is taken to mean that algal oxygen production is not sufficient to meet the requirements of the heterotrophic inhabitants of the envelope, and therefore could not provide a surplus to the embryo. It is suggested that the higher metabolic rates and more rapid development of embryos associated with algae must depend in part on some factor other than oxygen supplied by photosynthesis.

4. The problem of proving the existence of a growth factor is pointed out, and some additional unsolved problems regarding the life of the alga and its means of penetration into the egg envelope are indicated.

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A COMPARISON BETWEEN TASTE RECEPTORS AND OTHER NERVE TISSUES OF THE COCKROACH IN THEIR RESPONSES TO GUSTATORY STIMULI [1]

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It has long been known from behavioral studies that some butterflies, flies, and bees have taste receptors on the tarsi (Minnich, 1921, 1929, 1932). The electrophysiological studies reported here were begun to determine whether or not such tarsal taste receptors are present in the American cockroach, *Periplaneta americana*. In the course of these studies a clue was found to a fundamental characteristic of all taste perception in the cockroach. This is the similarity between the responses from recognized taste receptors and from nerve tissue unspecialized for taste perception. It is this relationship, briefly described earlier (Roys, 1956), which is the principal subject of this paper.

Materials

Adult male and female American cockroaches, *Periplaneta americana*, of various ages were used for all the electrophysiological experiments, while the behavioral experiments included nymphs of both sexes as well. Sex differences did not seem to have any effect on the responses in the experiments. They were all kept at room temperature and fed on powdered dog biscuit (Purina chow).

The chemicals used in the experiment were all of reagent grade except for the quinine which was U. S. P.

Nerve action potentials were picked up from the tarsal preparations through tungsten electrodes drawn to fine points in a gas-oxygen flame. The electrodes were connected by copper leads to a Grass P-3A amplifier and a Dumont 208B oscilloscope. A Grass cathode follower was also used in some of the experiments. In nerve cord preparations, bare silver electrodes were substituted for tungsten. Changes in nerve activity were measured with an Electrodyne decade impulse counter which recorded the number of nerve impulses per second. This was connected to the output of the oscilloscope amplifier.

Experiments and Results

Seven types of experiments were carried out—six on the response of various types of nerve preparations to a test substance and one on the behavioral response to the same substance presented in the drinking water. Four test substances were used—sodium chloride, hydrochloric acid, sucrose and quinine, corresponding to the four accepted taste sensations of salt, sour, sweet and bitter. First, sodium

[1] This work was made possible by a grant from the U. S. Public Health Service to Tufts University. Some of the apparatus used was obtained under a previous contract between the U. S. Chemical Corps and Tufts University.

chloride was used in all seven types of experiments, then hydrochloric acid, sucrose and quinine, making a total of 28 different experiments. Each of these 28 was repeated at least five times to insure the validity of the results. In the following sections A through G, the rationale, techniques and results of the experiments with sodium chloride are discussed in some detail. Section H deals with substitution of hydrochloric acid, sucrose and quinine for sodium chloride in these same experiments.

A. Intact tarsus

To test whether application of sodium chloride solutions to the tarsus would produce any afferent activity in the nerve of the leg, a prothoracic leg was cut off at the femoro-tibial joint and mounted with two electrodes in contact with the nerve. One was inserted into the opening at the cut end of the leg, the other pushed in through the membrane at the tibio-tarsal joint until the tips were about one millimeter apart within the tibia. The electrodes supported the leg, and the tarsus extended down into a wax cup filled with water or test solution which could be changed with a pipette. Because of the large diameter of the electrodes relative to the size of the tibia, they usually detected afferent impulses without special care as to their exact position. The tungsten electrodes were connected to the amplifier and oscilloscope.

When the tarsus was submerged in water in the wax cup, the nerve showed a steady discharge of typical nerve spikes which probably originated in mechanoreceptors of the leg. This basal activity was measured by counting the number of spikes per second with an electronic counter set to count all spikes above the noise level. Ten consecutive counts were taken in a group and averaged. When two or more successive groups of counts showed the same level of activity, it was considered that a satisfactory base had been established. Then the water was replaced with sodium chloride in successively higher concentrations of 1, 2, 3, 4 and 5 *M*. Each concentration was left in contact with the tarsus for approximately one minute. The nerve activity continued at about the same level until a sudden increase showed that the threshold had been reached, *i.e.*, that tarsal stimulation occurred at that concentration.

Selection of the "threshold" response must, from the nature of the experiments, be somewhat arbitrary, since the basal activity showed continual small fluctuations. To be certain that the threshold was a valid one, a point was selected where the activity clearly exceeded any of the preceding base line fluctuations or any probable instrumental changes caused by shifts in line voltage, etc. Usually when a clear increase of 100% or more in the number of spikes per second occurred immediately after a change in concentration of the chemical under test and lasted longer than the quick-adapting tactile response, it was considered to be the threshold point. In some instances lower percentage increases were accepted as thresholds due to special circumstances such as an unusually even base line. Experiments were continued until five or more such thresholds at the lower end of the range coincided within half a log unit of one another. The range of the five is given in Table I and the lowest one is plotted as the threshold indicated by the bar graph in Figure 1-A.

The threshold was usually reached at 5 *M* sodium chloride. However, it was

variable and in one instance occurred at a concentration as low as 1 *M*. The roaches used in this series of experiments were all adult females, but their age as adults was unknown. If the age was the variable factor, then it seemed possible that the work of Slifer (1950) on locusts offered a further explanation of this variation in thresholds. She found that water permeability of the cuticle on locust tarsi increased with age because the impermeable outer layer was abraded away in older individuals. This suggested that permeability of the tarsal cuticle might be the

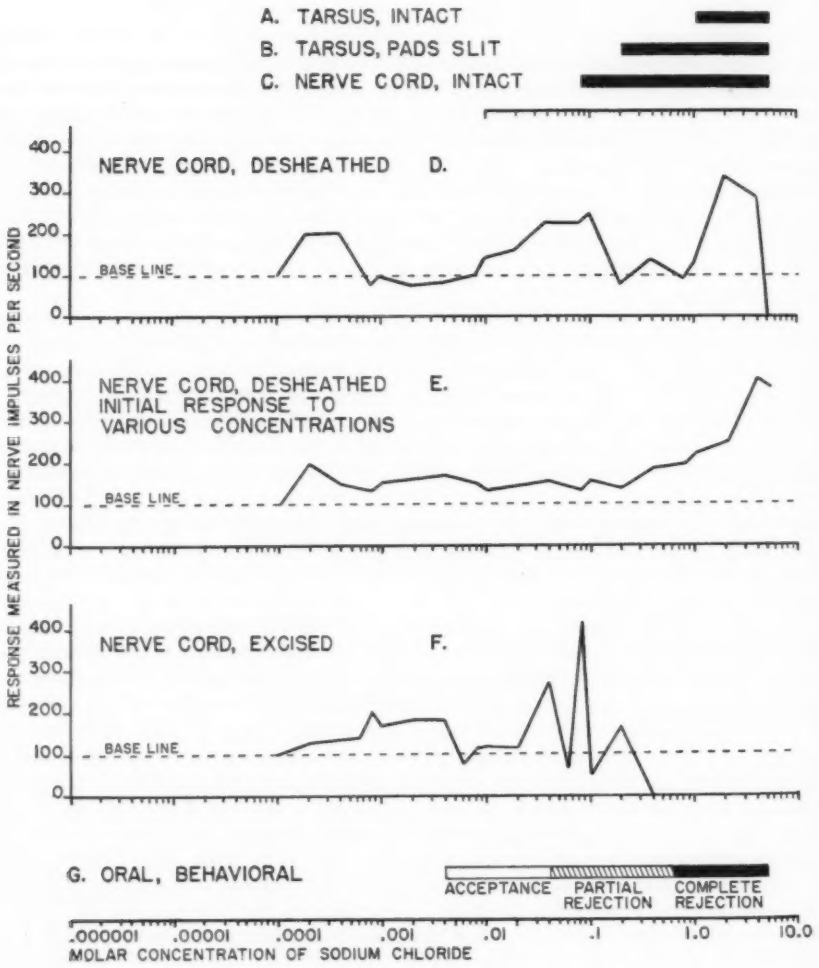

FIGURE 1. Responses of all types of preparations to sodium chloride

TABLE I

Thresholds for the four major taste qualities in different types of preparation

	Thresholds						Calculated osmotic pressure at slit tarsus thresholds
	Tarsus intact	Tarsus pads slit	Nerve cord intact	Nerve cord desheathed	Nerve cord excised	Behavioral oral	
Salt Sodium chloride	1–5 *M*	0.2–0.4 *M*	0.08–0.1 *M*	0.0002–0.0006 *M*	0.0002–0.0006 *M*	0.004–0.008 *M*	9.76 Atm.
Sour Hydrochloric acid	0.1–0.6	0.006–0.008	0.001–0.004	0.0002–0.0004	0.00001–0.00004	0.0006–0.0008	.29
Sweet Sucrose	No response at 2.5 *M*	0.1–0.4	0.1–0.6	0.002–0.004	0.001–0.004	0.006	2.44
Bitter Quinine	No response at 0.01 *M*	0.001–0.008	0.001–0.006	0.00008–0.0001	0.000006–0.000008	0.0002–0.0004	.02

factor controlling the tarsal thresholds in cockroaches. If so, slitting the tarsal pads to allow free entry of the test solution could be expected to lower the threshold.

B. Tarsus with pads slit

The tests were repeated using tarsi the five tarsal pads of which had been slit longitudinally with a razor blade. Concentrations for these experiments were increased by steps in accord with the following series: .01, .02, .04, .06, .08, .1, .2, .4, .6, .8, 1.0, etc. This progression of concentrations was also used for most subsequent experiments, but in some it was shortened to .02, .04, .08, .2, .4, .8, 2.0, etc., which approximates a doubling of concentration at each step. Because the test solutions would enter the tissue, they were made up in saline solution (9.0 g. NaCl, 0.2 g. KCl, 0.2 g. $CaCl_2$ per liter of solution; proportions from Pringle, 1938) instead of water. Thus in the sodium chloride of the test solution was included the amount of sodium chloride in saline solution plus the sodium chloride added. For example, at a threshold of 0.2 *M* sodium chloride in saline solution, the sodium chloride present was 0.15 *M* (from 9.0 g. per liter in the saline solution) plus the additional 0.2 *M*, giving a total concentration of 0.35 *M*. The same criteria for thresholds were used as for intact tarsi in the preceding series. The results are shown in Table I and Figure 1-B and it can be seen that the thresholds are much lower than for the normal intact tarsi.

C. Nerve cord in situ, intact

However, when the test solution entered the tissue of the tarsus it reached all types of nerve endings. Did the afferent response originate in special subcuticular taste receptors or in nerve endings unspecialized for taste? One way to answer

this question was to compare the responses of the tarsi to those from a nerve located where it could not normally be concerned with taste perception. Such nerves are found in the connectives and ganglia of the ventral nerve cord. If they respond to the same concentrations of sodium chloride it would indicate that the response of the tarsi was not dependent on special taste receptors.

It was found to be possible to pick up impulses from the ventral nerve cord in the intact roach simply by slipping the active electrode between the sternal plates and into the abdominal cavity just ventral to the abdominal cord. For routine experiments the roach was anesthetized with carbon dioxide, laid on its back on a small lucite block and secured in place with strips of Cenco Tackiwax. A bare tungsten electrode was inserted between the second and third abdominal sternites. The indifferent electrode was inserted in the opening formed by cutting off the tip of an antenna. Since the roach antenna contains no muscles or efferent nerves, an electrode placed there does not pick up any extraneous nerve or muscle potentials. The test solution was injected close to the cord and posterior to the pickup electrode with a fine glass pipette. Base line and threshold were established as before and the thresholds obtained are shown in Table I and Figure 1-C. They are slightly lower than those from the tarsi, indicating that the tarsal response is not entirely dependent on special taste receptors.

D. Nerve cord in situ, desheathed

Twarog and Roeder (1956) showed that the sensitivity of the roach nerve cord to chemicals was greatly increased when the connective tissue sheath was removed. To test the effect of this sheath on the sodium chloride threshold, the experiments were repeated using a modification of the Twarog window preparation. For this preparation the head and hind legs of the roach were removed, and the roach laid on its back on a plastic block and held in place by a band of Tackiwax across the thorax, leaving the ventral side of the abdomen exposed. With a fine pair of scissors two windows were cut in the ventral abdominal wall. For one, the central section of the second sternite was cut away to expose the ventral nerve cord. A loop of the exposed cord was lifted out and hung over a bare silver wire which formed the pickup electrode. As soon as the nerve cord had dried enough to stick to the wire a little, it was cut on the anterior side of the electrode to limit activity to that originating in the abdomen. When a cathode follower was used with this type of preparation it was possible to let the exposed section of nerve cord dry completely, the pickup then being through the dry dead section to the living cord inside the body (Roeder and Treat, 1957). The indifferent electrode was inserted into the body cavity at the neck. The second window was formed by cutting away the central portion of the fourth, fifth and sixth sternites to expose a nerve cord ganglion and its adjacent connectives. Under this section of cord was placed a narrow strip of Parafilm or wax paper to separate it from the underlying tissue and body fluid. The connective tissue sheath was torn with fine forceps (see Twarog and Roeder, 1956) and the exposed section perfused with saline followed by a series of test solutions. The perfusing fluid was supplied from a small reservoir through a fine glass tube and carried away by a wick of absorbent paper.

The results are shown in Table I and Figure 1-D. The base line of this and all

subsequent curves is adjusted to 100 for convenient comparison with other experiments. Curve D is taken from a single experiment considered typical of those done. The salient peaks and hollows were found in curves from all the experiments of the series, but they varied in their exact position on the x-axis so that an addition of several curves to form a composite would conceal the true form of the curve through cancellation. Hence, the single representative curve is given instead of a composite based on several experiments.

It will be noted that the thresholds are much lower than those of either intact sheathed cord or tarsus, clearly showing that the sheath checks penetration of sodium chloride and conversely that activity stimulated by these low concentrations must come from within that part of the cord normally enclosed by the sheath.

E. Nerve cord in situ, desheathed. Initial response to various concentrations

The preceding curve D, showing the response to steadily increasing concentrations of sodium chloride, has a characteristic succession of peaks and depressions. On first thought, one might interpret this to mean that the nerve does not respond to certain concentrations of sodium chloride, *e.g.*, between .0008 *M* and .008 *M*. However, it seemed more likely that these depressions represented some sort of adaptation to continued exposure to sodium chloride, and that a fresh nerve cord would respond initially to any concentration of sodium chloride above the threshold value of .0002 *M*. To check this a series of experiments was run to determine the response of a freshly dissected nerve cord in saline to each concentration shown on the curve. The results are shown in Figure 1-E. At the highest concentrations, blocking of all nerve activity began before the ten counts were completed so that the end of the curve, based on an average of ten readings, shows a drop even though the first one or two readings are higher than any preceding ones.

It can be seen from the figure that there are no concentrations above threshold to which the nerve does not initially respond, and that the extent of response generally increases with the concentration of sodium choride, particularly at high concentrations. This seems to support the inference that the depressions in curve D are due to the cumulative affect of previous treatment and do not result solely from the concentrations at which they appear.

F. Excised nerve cord

While the experiments with the normal and desheathed cord showed a clear response to rather low concentrations of sodium chloride, it may be questioned whether this response was due to direct action on the nerve cord. Possibly the perfusing fluid leaked down onto some unsuspected area of taste receptors. To check this possibility the abdominal section of the nerve cord was removed from the roach and tested alone. This consists of a chain of six ganglia joined by paired connectives and is about one centimeter long. It was dissected out and laid across silver wire electrodes in a small depression in a lucite block, in a modification of the preparation described by Roeder and Roeder (1939). When this depression was filled with saline or test solution, the cord was submerged, and no impulses were picked up because of electrical shunting through the saline solution. However, when the solution was drawn out of the depression with a small piece of absorbent

paper, the moist cord was left hanging in the air across the two electrodes and impulses were picked up for viewing on the oscilloscope and for counting.

To establish the base line activity of the cord in saline solution, it was first submerged in saline for several minutes to equilibrate with the new medium, then the solution was drawn off, ten counts of the number of spikes per second taken, and the cord submerged in saline again for one minute before another count was taken. As soon as the base level of activity had been established as for the intact tarsus, test solutions of increasing concentrations of sodium chloride were substituted for pure saline. Thus the cord was alternately submerged and exposed for periods of one minute each during the experiments, in contrast to the preceding experiments where the nerve was continually perfused. However, length of exposure to each test solution was approximately the same in both types of experiment.

Although the excised nerve cord was not desheathed, numerous openings were left wherever connectives were cut away and where the whole cord was cut at the anterior end in the process of excision. Therefore, it seems probable that the protective function of the sheath was reduced almost as much as by stripping it away.

In this preparation all six ganglia and their connectives were simultaneously exposed to each change of solution, in contrast to the preparations with the nerve cord *in situ* where only a single ganglion and its connectives were exposed. This produced responses in the excised cord which were more sharply defined and the thresholds were more easily determined than with the cord *in situ*. It also resulted in blocking at a lower concentration than that which blocked the cord *in situ*, where some of the ganglia and connectives were protected from direct exposure to the salt.

From Table I and Figure 1-F it is clear that the threshold is at least as low as that of the cord *in situ*, further confirming that no special taste receptors are needed to account for the response. Considering the preceding six types of experiment in retrospect, it is now clear that as the protective coverings are stripped away—first the cuticle to expose the leg nerve and intact cord to chemical action, then the sheath from the exposed cord—the sensitivity increases. Receptors specialized for taste are usually assumed to be more sensitive to chemicals than other nerves. However, in view of the low threshold of the desheathed nerve cord it seemed worth while to check the sensitivity of recognized taste receptors for comparison.

G. Oral taste, behavioral

Evidence from various sources and confirmed by Frings and Frings (1949) indicates that the maxillary and labial palpi of the cockroach carry the oral taste receptors. Therefore the palpi were set up in the same way as the tarsal preparations, in the expectation of getting responses in line with the behavioral thresholds reported by Frings (1946). However, the thresholds obtained were only slightly lower than those from the intact tarsi. It seems probable that this discrepancy was due to the very small size of the fibers which carry the normal gustatory responses —a condition which makes recording very difficult. To avoid this difficulty the method developed by Hodgson, Lettvin and Roeder (1955) was tried. Working

with flies they were able to make electrical contact with single sensory hairs in the oral region which responded clearly to chemical stimuli. However, this method proved unsuccessful when applied to the cockroach. The longer hairs at the tips of the palpi did not give any clear responses to gustatory stimuli, and it may be that gustatory perception is through short bristles or pegs which lie between the longer hairs and are therefore rather difficult to reach with the electrode. Therefore, for the present we must rely on the older method of behavioral response.

Behavioral thresholds for sodium chloride were determined by a modification of the method used by Dethier and Rhoades in 1954 for flies. This method offers the test population a choice between flavored and unflavored drinking water and measures the amount of each kind consumed in each of a succession of trial periods. In each trial the concentration of flavored material is greater than in the preceding one. Any change from a one-to-one ratio of consumption indicates ability to distinguish between the two, *i.e.*, the threshold concentration of the flavoring material.

In the first of these experiments a colony of about 100 adult roaches of both sexes was used for one series of experiments and a colony of nymphs for another series. However, no differences between the responses of nymphs and adults were noted and in subsequent experiments a breeding colony of mixed nymphs and adults of both sexes was used. The roaches were confined in 15-gallon aquaria containing cardboard shelters, but were not restricted in any other way and were free to eat and drink whenever they chose. Temperature ranged from 22° to 26° C. and the cages were lighted during the day by room illumination and were dark at night. This near-normal environment and lack of any restriction to movement of the experimental animal are notable advantages of this method.

The drinking water for each colony was supplied from two identical glass tubes of 6 mm. inside diameter and about 50 cm. long. These tubes lay parallel on the bottom of the aquarium except for the ends which were bent up to prevent the water from running out. At one end the bent sections came up at right angles for 12 cm. and were taped to the wall of the aquarium to hold them in position. The other two ends sloped up at 30° to a height of 1.5 cm. above the aquarium floor and were plugged with rolled cylinders of lens paper which acted as wicks to draw the water from the long horizontal reservoirs and make it available to the roaches. It was found best to put the ends of the tubes with the wicks about one centimeter apart, fastening them to a spacer block with Tackiwax to hold them firm. They were in an open area of the aquarium floor and in the light. The lens paper plugs were changed daily. Each day each tube was filled with water from a graduated syringe to a mark 1.5 cm. up on the vertical section. Thus the amount of water consumed in the preceding 24 hours was determined by measuring the amount needed to refill the tube to the mark. A colony of 100 roaches took about 10 ml. of water a day or 5 ml. from each tube if the tubes were equally preferred. Evaporation, checked in a separate tube, was nearly constant at 0.5 ml. per day. Day-to-day fluctuations in consumption from the two tubes were erratic and ran as high as 20% difference between the two with water in both tubes. To determine the salt threshold, increasing concentrations of sodium chloride (in the same steps used in the nerve preparations) were substituted for water in one of the tubes. The threshold concentration was clearly marked by a sharp increase in preference for the tube containing sodium chloride, *i.e.*, an acceptance threshold.

This was followed by a continued preference for the sodium chloride tube at higher concentrations until the rejection threshold was reached when there was a sharp change in preference from sodium chloride to water. Higher concentrations of salt were progressively less and less acceptable until a concentration was reached at which no salt solution at all was taken, *i.e.*, salt was completely rejected at or above that concentration. Supplementary experiments showed that previous conditioning had little effect on any given trial. For example, in a given pair of tubes when an unacceptable sodium chloride solution was replaced by water, there was an immediate return to an approximately one-to-one ratio of preference and the same appeared to be true in shifting from an acceptable solution to water.

Two complete series, ranging from well below the acceptance threshold to well above the point of complete rejection, were run. In addition three short series were run in the ranges of the acceptance and rejection thresholds. The results are shown in Table I and the bar graph in Figure 1-G, and it is clear from these that the behavioral threshold is well above that of any of the nerve cord preparations.

H. Responses from all types of preparation to sour, sweet and bitter stimuli

To test whether comparable thresholds and curves could also be obtained from sour, sweet and bitter substances, similar series of tests were carried out with hydrochloric acid, sucrose and quinine. Quinine monohydrochloride was substituted for quinine at concentrations above 0.001 *M* because of its greater solubility. At lower concentration it had the same threshold as the pure alkaloid. The experiments with sucrose and quinine were not carried out to 5.0 *M* because sucrose solution becomes a thick syrup above 2.0 *M* concentration, while quinine monohydrochloride tends to precipitate out of solution at concentrations much above 0.01 *M* in water. In every other way the same procedure was followed as for sodium chloride.

The results of these experiments are shown in Table I and Figures 2, 3 and 4. Figure 5 is a summary of all the thresholds for all four substances, plotted on a single graph for comparison.

In comparing the responses to hydrochloric acid with those to sodium chloride it is at once apparent that most of the hydrochloric acid thresholds are lower. An apparent exception is found in the thresholds of the desheathed nerve cord *in situ*. This discrepancy is probably due to a limitation of technique rather than a real physiological difference. This technical limitation lies in the fact that much less area is exposed to test solutions with the cord *in situ* than with it excised (see section F). For this reason the responses from the cord *in situ* were less clearly defined and the threshold less easily determined. In the cases of sodium chloride and sucrose the experimental evidence seemed to support thresholds of the cord *in situ* as low as those of the excised cord. With hydrochloric acid and quinine the evidence at hand did not seem to warrant a firm statement to this effect, although there is a strong probability that it is true for these substances also.

As with sodium chloride, the behavioral tests with hydrochloric acid showed both acceptance and rejection thresholds, and concentrations causing rejection also stimulated the tarsi and produced a strong response in the nerve cord.

Sucrose did not stimulate the intact tarsus in concentrations up to 2.0 *M*, and

thresholds for all types of preparation were rather high. In the behavioral tests sucrose was acceptable at all concentrations from threshold to 2.0 *M*.

Quinine also failed to stimulate the intact tarsi, suggesting that the cuticle is less permeable to the large molecules of sucrose and quinine than to the ionized salts and acids. In behavioral tests quinine was rejected in all concentrations above the threshold.

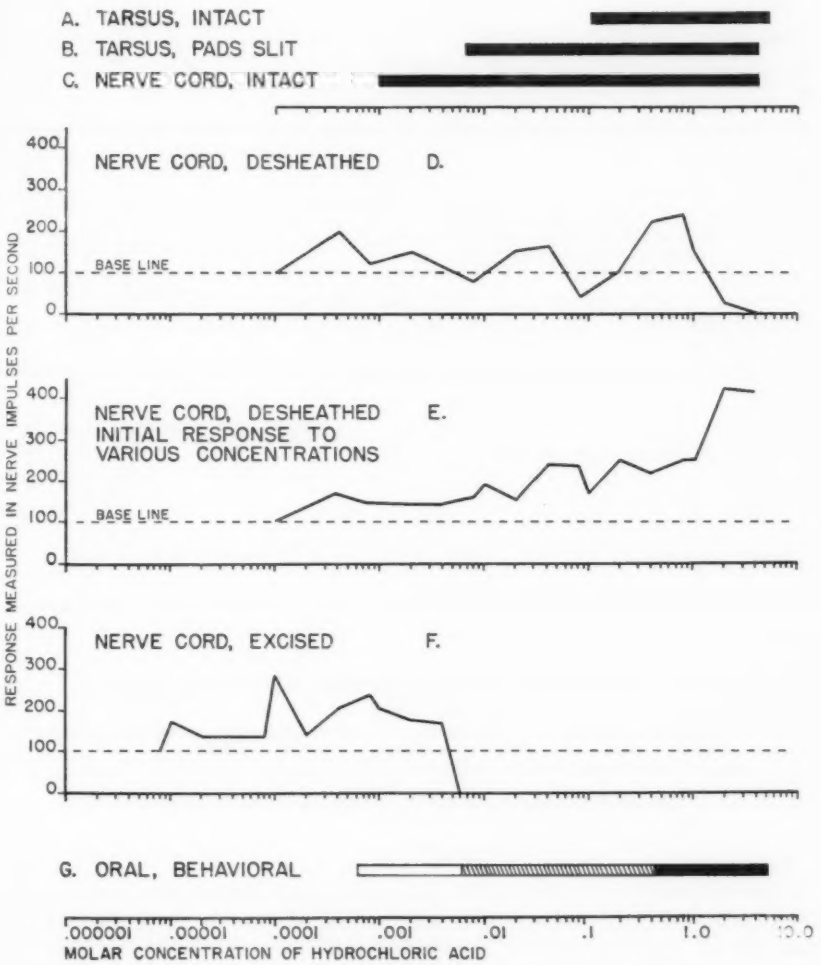

FIGURE 2. Responses of all types of preparations to hydrochloric acid. Legend for bar: unshaded area, acceptance; cross-hatched area, partial rejection; black area, complete rejection.

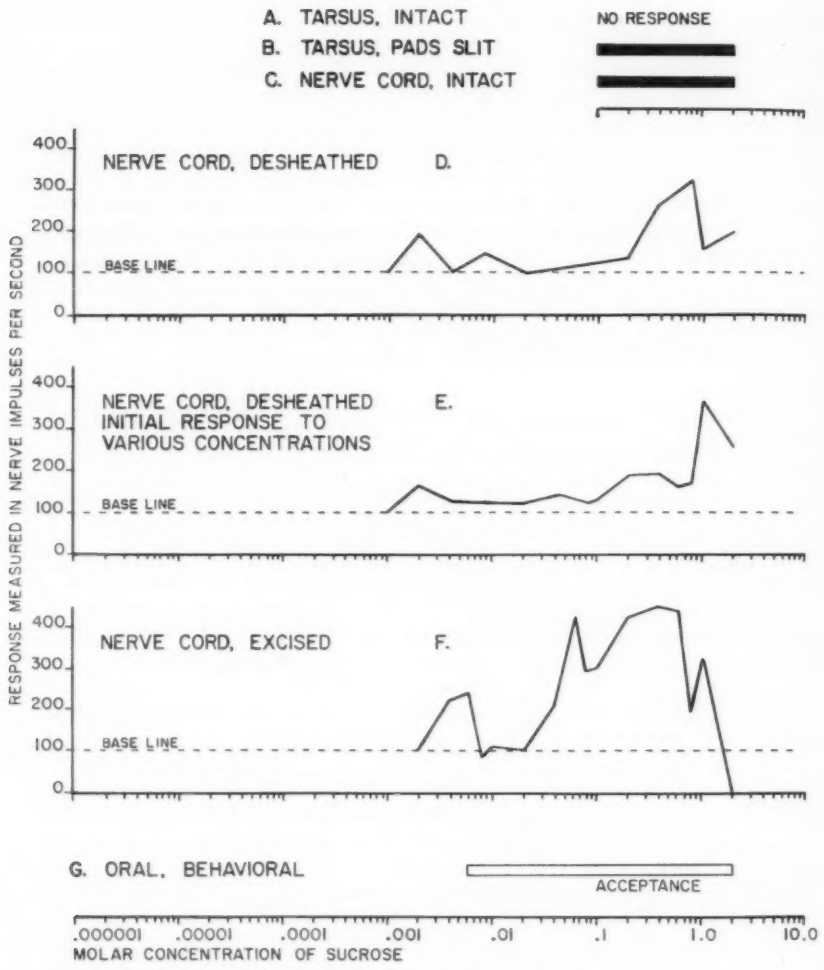

FIGURE 3. Responses of all types of preparations to sucrose.

DISCUSSION

The foregoing sets of experiments seem to indicate that the chemical sensitivity of recognized oral taste receptors, as well as receptors on the tarsi, is always less than that of many other nerves in the body not normally concerned with taste. However, there are a number of points in the work which may need clarification or can profitably be amplified.

The first consideration is whether the nerve responses obtained are true chem-

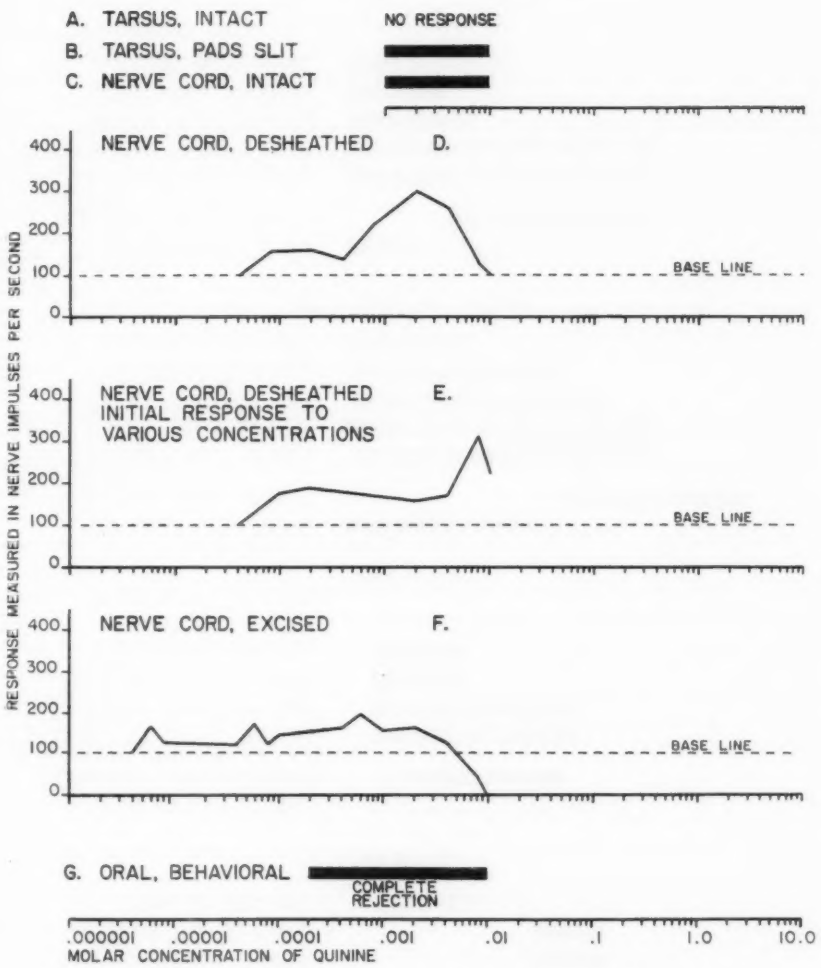

FIGURE 4. Responses of all types of preparations to quinine.

ical thresholds or whether they are due to osmotic or other physical changes. If they are due simply to increased osmotic pressure, then, conversely, threshold concentrations of all four substances should produce the same osmotic pressure. However, the computed osmotic pressures at threshold concentrations for sodium chloride, hydrochloric acid, sucrose and quinine are given in Table I and show a wide range of values for the four substances. This would seem to show conclusively that osmotic pressure was not the principal cause of the responses. The wide range

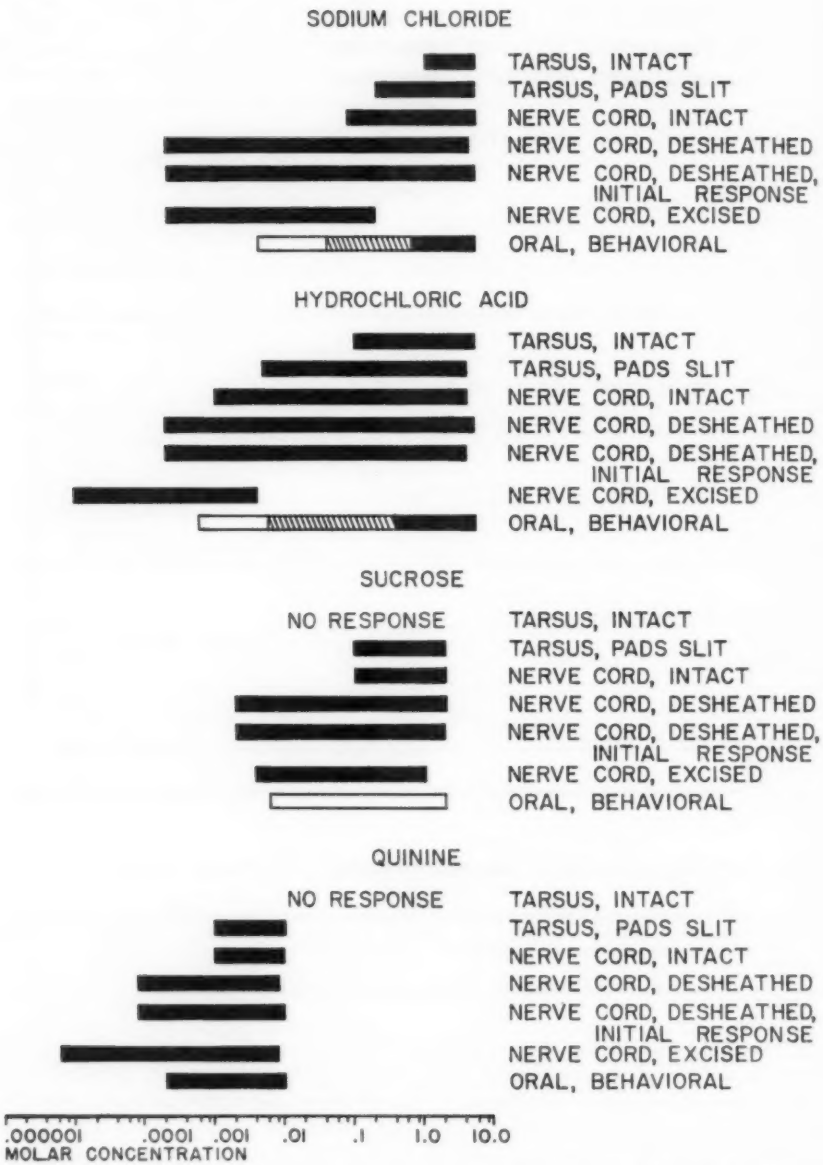

FIGURE 5. Responses of all types of preparations to sodium chloride, hydrochloric acid, sucrose and quinine

over which the thresholds extend seems to be further evidence against a single physical factor being responsible.

Secondly, some may question the physiological validity of certain of these thresholds. In particular the response of the nerves to sucrose may seem to be in direct conflict with the widespread practice among neurophysiologists of using "inert" sucrose to maintain the osmotic pressure of physiological saline solution in the absence of certain salts. However, it should be emphasized that at threshold concentrations the effects of these solutions are transitory. It is customary to allow a short period of time for any nerve preparation to recover from injuries of surgery, small osmotic changes produced when saline solution is substituted for blood, etc., before the activity is studied. Thus these transitory threshold responses might easily be overlooked or masked by other adaptive changes in activity. However, they show up clearly when separated experimentally from other possible causes of nerve activity. Moreover, when used in adjusting osmotic pressure the sucrose is substituted for some constituent of the solution, not added to the solution as was done in these experiments.

It is also possible that injury from operative techniques may have made the nerves more sensitive to chemical action. This seems unlikely in the tarsal preparations where injury was minimal and in the normal nerve cord where test substances were injected into an otherwise intact animal. Also, the Twarog window preparation was particularly designed to do a minimal amount of damage, and this is supported by the fact that thresholds for nerve cord exposed in the window with the sheath intact were no lower than those obtained by injection of the same substance. Desheathing may have torn some of the nerve fibers, but it seems very unlikely that these could have amounted to more than a small fraction of the total which responded to threshold concentrations. However, in the excised nerve cord a large number of connecting fibers were cut to remove it and these cut ends may have been very sensitive. This may have been a factor in the very low thresholds obtained from this type of preparation in response to stimulation by hydrochloric acid and quinine. If so, this would be interesting in itself, but these two thresholds are not essential to the general thesis of this paper.

Another important consideration is whether or not the thresholds from the intact tarsi and from exposed nerves are truly comparable to oral taste. The strongest argument in favor of this is the close correlation between the behavioral threshold and the thresholds of the various nerve preparations in response to a given chemical. This is most clearly shown in the responses to sucrose and quinine (see Figure 5) where the thresholds of the various nerve preparations for quinine are much closer to the behavioral threshold for quinine than to the behavioral threshold for sucrose and vice versa. The same may be said for responses to any other pair of test chemicals though it is more conspicuous in some pairs than in others.

Further correlation is found between the behavioral rejection thresholds for sodium chloride and hydrochloric acid and the activity produced in the nerve preparations by the same concentrations of the salt or acid. These concentrations suffice to raise the activity in desheathed nerves to a very high level and initiate activity in those nerves protected by a sheath or even by a sheath plus cuticle. This correlation seems to lend additional support to the relation between behavioral responses and the responses from nerve preparations. Further, it suggests that

when the stimulating chemical reaches a concentration which produces violent activity in the special taste receptor and begins to produce activity in all other nerves in the area, behavioral rejection sets in.

Results from the experiments with quinine do not show this correlation as clearly, while with sucrose we find violent activity in the nerve cord correlated with behavioral acceptance. However, this need not detract from the significance of the correlation between behavioral rejection and nerve response with salts and acids, since it is probable that the bitter and sweet molecules act on the receptors through quite different mechanisms than the salt and acid ions.

The protection afforded by the sheath in these cases brings us to a general summary of the factors which determine thresholds in different types of preparation. In the leg nerve preparation the tarsal cuticle was ruptured with the result that a lower concentration of chemical was needed to initiate nerve action. However, the sheath remained as a second line of protection. It was not practical to remove the sheath from the leg nerve, but a switch to the nerve cord showed similar thresholds. When the nerve cord sheath was removed, the concentration required for stimulation again dropped markedly. Viewing these experiments in general terms, it now seems probable that the *true* nerve threshold is the concentration of test substance which, when acting directly on the unprotected nerve, will produce a response. Any higher thresholds for protected nerves are measures of the concentration which must be applied outside the sheath or cuticle to produce the *true* threshold concentration at the nerve. Extrapolating to the normal oral taste receptors which control the behavioral thresholds, we may infer from the position of these behavioral thresholds, intermediate between those of sheath-protected and of desheathed nerves, that the nerves which govern them are not enclosed by normal sheath or cuticle but have more protection than bare nerve. This barrier may be to protect them from damage by high concentrations of chemicals, or it may be concerned with selection or differentiation between different types of taste stimulation.

In examining the curves lettered D and F, which represent the responses of desheathed nerve to the four chemicals tested, the most striking characteristic is the sharp drop in activity first seen after the threshold response. This rise to a peak of activity followed by a period of depression is repeated one or more times in varying degrees in all the curves before blocking occurs. A possible explanation is that the three or more peaks, particularly clear in the excised nerve cord preparations, may come from different groups of fibers each less sensitive or less exposed than the preceding. Each of these groups in turn could become active, reach a maximum, and then adapt or partially block to account for the peaks and depressions as the concentration of stimulating chemical is increased. In other words, parts of the nerve cord may be protected from chemical action by barriers comparable to the connective tissue sheath and the cuticle.

It is interesting to compare the behavioral thresholds with those obtained by Frings (1946) who also worked with cockroaches, using a different test method. In testing whether individual roaches would accept or reject test solutions offered them, Frings first determined the sucrose threshold by offering increasing concentrations of sucrose in water until an acceptable concentration was reached. This acceptance threshold was clear, but since individual roaches often refuse pure water, he had to use a different method to determine rejection thresholds. For this he

TABLE II

Behavioral thresholds determined by two methods

Thresholds	Sucrose	Sodium chloride			Hydrochloric acid		
	Acceptance	Acceptance	Rejection		Acceptance	Rejection	
			Partial	Complete		Partial	Complete
Reported here	.006	.004	.04	.6	.0006	.006	.4
Frings	.007			.2			.02

selected a sucrose concentration far enough above threshold to be always acceptable (0.1 *M*) and offered this with the addition of increasing concentrations of sodium chloride until it was rejected. This he took as the rejection threshold for sodium chloride. He repeated the experiments with hydrochloric acid and a large number of other salts and acids, and Table II shows a comparison of his results with the behavioral thresholds determined in these experiments.

It is at once apparent that there is very good agreement on the determination of sucrose against water, but the present method gives much more information about the sodium chloride and hydrochloric acid. As might be expected, Frings' lowest records for rejection lie within the range reported here as partial rejection. Frings was aware of the existence of the lower acceptance thresholds reported here but was unable to determine them because he could not get consistent feeding responses without the use of sucrose. It was the use of a large population of roaches instead of individuals which made it possible to get consistent feeding responses in these experiments without the use of sucrose.

It is quite possible that these thresholds as well as those of the nerve preparations may vary with different diets. In these experiments the same diet was supplied throughout, but it seems probable that any dietary change would affect both behavioral and nerve thresholds equally so that the same relationships would remain.

The methods used do not show whether the impulses from the nerve cord arise in the axons, dendrites or somata of the activated nerve cells. Since all impulses above the amplifier noise level were counted, it is not possible from the present data to estimate either the size of the responding elements or their relative number in the total population of active neurons. From other studies (Roeder, 1948) it seems probable that many compounds exert their effects on the dendritic or somatic regions of the central neurons.

These studies seem to indicate that the chemical thresholds for nerves unspecialized for gustatory reception are as low as, or lower than, those of the specialized receptors. On this basis we may conclude that taste or contact chemoreception depends on two factors. One of these, sensitivity, is also held by the neurons unspecialized for gustatory perception. Therefore, we can study it in nerve tissue other than the complex receptors which offer considerable technical difficulty. Furthermore, we can bring to bear on this study the vast amount of work which has previously been done on nerve tissue. The other quality, discrimination be-

tween different substances, remains a property of the receptor mechanism. However, it is hoped that its study has been simplified slightly by separating from it the factor of sensitivity which has often been considered an integral part of this mechanism.

Summary

1. Application of increasing concentrations of sodium chloride to the normal intact tarsi of the American cockroach resulted in increased activity in the afferent fibers of the leg nerve when the threshold concentration was reached.

2. The threshold for this response was lowered by slitting the tarsal pads.

3. Although it may be presumed that there are no taste receptors on the nerve cord, when increasing concentrations of sodium chloride in saline solution were applied to an exposed section of intact nerve cord, it responded to a lower concentration than did the tarsal preparations.

4. The threshold of the nerve cord was further lowered by removing the connective tissue sheath which normally encloses it.

5. A section of the nerve cord, completely removed from the roach and exposed to the same concentrations of sodium chloride, responded at the same threshold concentration as the exposed nerve cord *in situ*, showing conclusively that the response did come from the nerve cord itself, not from adjacent chemoreceptors.

6. Behavioral experiments showed a response to the taste of sodium chloride at a threshold *higher* than that of the nerve cord preparations.

7. There was also an increase in the nerve activity from leg and nerve cord preparations in the same range of concentrations of sodium chloride which produced behavioral rejection.

8. Similar experiments with hydrochloric acid, sucrose and quinine, representing the sour, sweet and bitter sensations, also showed behavioral thresholds higher than those from the nerve cord preparations, and hydrochloric acid showed a correlation between nerve activity and behavioral rejection similar to that of sodium chloride.

9. It was concluded that high sensitivity to the four types of substances which produce the four taste sensations is inherent in nerves not normally connected with taste rather than being a special feature of the taste receptor, and that the basis for behavioral rejection may also be found in nerves not normally concerned with taste.

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HISTOPHYSIOLOGICAL STUDIES ON THE CORPUS ALLATUM OF LEUCOPHAEA MADERAE. I. NORMAL LIFE CYCLE IN MALE AND FEMALE ADULTS [1]

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In the course of previous work in our laboratory dealing with the functional and cytological properties of neuroendocrine systems, in particular the intercerebralis-cardiacum-allatum system of the insect, *Leucophaea maderae* (Scharrer, 1946b; Scharrer and Scharrer, 1944), it was observed that the corpora allata display an impressive variability in their morphology. The question arose whether differences in the size and histological appearance of these glands reflect differences in age, sex, functional state, etc., as is the case in an analogous organ, the anterior pituitary, or merely constitute the range of individual variation, or perhaps both.

The first indication that physiological state determines structure in the corpus allatum of *Leucophaea* was obtained by an analysis of the results of experiments in which nerves connecting this organ with the brain were severed. Following this operation, when performed at the appropriate time, the functional capacity of the gland was stepped up as demonstrated in last instar nymphs (Scharrer, 1946a), and there was a marked increase in glandular volume and relative cytoplasmic content (Scharrer, 1952). The conclusion seemed justified that, at least under the conditions of these experiments, a large gland in which the nuclei are loosely distributed represents a physiologically active gland.

This conclusion was borne out further by more recent studies (Engelmann, 1957) describing cyclic changes in the size of the corpora allata of normal adult females of the same species, *Leucophaea maderae*, in conjunction with reproductive processes.

Another investigation in which corpus allatum volume could be correlated with functional change concerns the effect of gonadectomy (von Harnack and Scharrer, 1956). These observations gave rise to two questions: (1) Are the increase in the size of the corpus allatum following gonadectomy and that occurring after nerve severance unrelated though comparable phenomena, or is there a mechanism involved which operates in both instances, resulting in a similar histophysiological response? (2) Is the volumetric increase observed in the corpora allata after both types of operation equivalent to that occurring under normal physiological conditions, *i.e.*, merely a sign of "activation," or does the response of the operated specimens go beyond the normal physiological range characteristic of the corpora allata?

In order to answer these questions data are needed, in addition to those reported by Engelmann (1957), concerning variations in the morphology of the corpora

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[2] Grantee of the American Association of University Women.

allata in conjunction with physiological states throughout the adult life span of both males and females. Therefore, prior to reports on experimental results, the present paper deals with cytological evidence of secretory activity and variations in cell size, cell number, and nuclear-cytoplasmic ratio in the normal animal to the extent to which they can be examined with conventional techniques of light microscopy. These observations will serve as points of reference for subsequent papers which will deal with experimental observations.

Material and Methods

The material on which this investigation is based consists of 88 female and 46 male normal animals of known adult age. They were removed from stock colonies on the day of their emergence and isolated in pairs in pint-size jars. They were kept at room temperature on a routine diet of dog chow and fresh apple until the day of fixation. The animals were killed at selected intervals ranging from 0 to 599 days in the female, and from 0 to 471 days in the male series. In the majority of cases an autopsy was performed to determine the condition of the internal organs, particularly that of the reproductive organs of the females.

For the histological study of the corpora allata and associated endocrine organs, the heads were fixed in Zenker-formol. Among a variety of staining techniques tested the aldehyde fuchsin method (Gomori, 1950) proved most useful, when modified according to Halmi (1952) and Dawson (1953). The addition of Weigert's hematoxylin as a nuclear stain permitted the use of the sections for nuclear counts as well as a study of secretory products. Most of the tissues were cut serially at 7 μ, a small number of cases at 5 μ.

As a basis for comparison of the morphological characteristics of the corpora allata, three values were determined: the volume of both glands, the total number of nuclei, and their relative number per unit of tissue (nuclear-cytoplasmic ratio). In addition, nuclear size was measured in representative cases of large and small glands. Since it was considered desirable to include a large number of animals in the normal, as well as the experimental series which will be treated in subsequent papers, it became necessary to select methods of determining these quantitative values which afford a sufficient degree of accuracy without excessive expenditure of time.

(a) Determination of volume

The conventional method of estimating organ volume by measurements of each consecutive section in a series, when carried out in hundreds of specimens would be a staggering task. Therefore, methods based on the measurement of representative sections were explored for their validity in expressing organ volume. This seemed feasible because in our studies the aim was not so much to determine glandular volume as accurately as possible, but to select a convenient and reasonably valid numerical expression of quantitative differences. An additional advantage of such a simplified procedure is the possibility of using incomplete series of histological slides, a not infrequent result of the technical difficulties encountered with chitinous material.

The validity of the method to be adopted was tested as follows. To serve as a basis for comparison, the volumes of the corpora allata of 20 representative cases

were calculated in μ^3 as accurately as possible by drawing each consecutive section with the aid of a camera lucida and measuring the drawings with a planimeter. By the inclusion of the extremes in this group of cases the range of variability was accurately established. Next, the same 20 cases were evaluated by measuring only intermittent sections. A comparison of results showed inaccuracies to be still rather small when only every ninth section was measured. The average number of sections per corpus allatum being 40 to 50, approximately 6 measurements were available for the calculation of the volume of each gland. Estimated values obtained by calculating the means between each of two consecutive measured sections were substituted for the values of the "skipped" sections. The inaccuracy of this procedure when compared with the results of measuring every section turned out to be small enough to permit the adoption of the abbreviated method for this and the following papers of this series.

(b) Nuclear counts

An accurate determination of the density of nuclear distribution meets with certain difficulties especially in small corpora allata poor in cytoplasm. In addition to the crowding of nuclei, a certain degree of irregularity in nuclear distribution has to be taken into consideration. Different methods of estimation were tested; the most reliable figures were obtained by direct counts under the microscope with the aid of a micrometer disc added to the eyepiece and of a tally counter. In each corpus allatum three representative sections were selected in approximately the same location, *i.e.*, the largest section in the middle of the series plus the fifth section from the middle section on either side. The total area of the three sections representing each corpus allatum and the sum of nuclei counted in this area permitted the calculation of the number of nuclei per mm.^{2}, a figure which represents a fairly good index of nuclear density. These values are recorded in Figures 1 and 2.

In order to determine whether a volumetric increase in the corpora allata is due solely to a rise in cytoplasmic content or whether this is accompanied by an increase in nuclear number, an at least rough calculation of the absolute number of nuclei present in each pair of corpora allata studied became desirable. For this the following formula suggested by Engelmann (1957) was used:

$$N = \frac{N' \times V}{A \times (T + 2r)},$$

where N = total number of nuclei per pair of corpora allata, N' = number of nuclei counted in A (total area of three sections selected for nuclear counts), V = volume of both corpora allata, T = thickness of sections, $2\ r$ = average nuclear diameter.

Results

a. Females

The aim of this study was to analyze in detail the periodic changes in the corpora allata in the course of one reproductive cycle, and to determine whether essentially the same pattern obtains in successive cycles throughout the adult life span. It soon became apparent (and was subsequently substantiated in our

TABLE I

Quantitative changes in the corpora allata of 26 females of Leucophaea maderae of varying adult age (maximum 599 days) grouped according to different stages in the reproductive cycle

	Number of animals	Range in volume of corpora allata (in mill. μ^3)	Range in number of nuclei	Range in nuclear-cytoplasmic ratio (nuclei/mm.^{3})
Group A (small eggs in ovary; beginning of cycle)	8	5.0–10.8	3657–6411	733–2034
Group B (growing eggs in ovary)	3	9.0–12.4	4137–9488	864–1558
Group C (pre-ovulatory stage)	3	15.3–37.0	7017–15,591	825–987
Group D (pregnancy)	12	3.9–17.3	3124–11,361	1155–1978

experimental work) that the corpus allatum of *Leucophaea* shows a fair degree of structural variability within groups of specimens selected under comparable physiological conditions. This fact had to be taken into account in the search for significant correlations with functional states and necessitated the use of larger samples than would otherwise be called for.

There is also a certain degree of individual variation in the timing of the alternating periods of ovarian activity and quiescence characteristic of the reproductive activity of *Leucophaea*. Therefore, an analysis of the first cycle which begins after the emergence of the adult offers advantages because of the greater ease of dating. For the study of subsequent cycles the "adult age," *i.e.*, the time elapsed since emergence, is less significant than the conditions of the ovary at the time of fixation. Consequently the selection of appropriate stages in these later cycles is facilitated by the recording of preceding parturitions. This is taken into account in the arrangement of the data summarized in Figure 1. These include 62 females killed at intervals of a few days during the first and second reproductive cycles. The cases illustrating the first cycle are arranged according to adult age (days elapsed between emergence and fixation of the animal), those of the second cycle are grouped in reference to the interval between preceding (*i.e.*, first) parturition and fixation.

An additional 26 animals studied in this series encompass the remainder of the entire adult life span. The oldest specimens represent extremes in longevity obtained from a large collection of dated females and are of particular value in the search for possible changes in corpus allatum function with increasing age. This group of older females, dated according to adult age, does not lend itself for the same graphic representation as the younger specimens, since the number of reproductive cycles they had completed was not recorded in all cases. This group is, therefore, not included in Figure 1. The values obtained for these animals grouped according to phases of the reproductive cycle are summarized in Table I.

As can be seen from Figure 1, within 30 days after emergence the volume of the corpora allata rose from an average value of 4.2 million μ^3 (minimum 3.5) in the very young female to an average value of 15.3 million μ^3, (maximum 16.6) corresponding to the time when the largest oocytes had almost reached their maximal size.

After ovulation, the corpora allata returned to approximately the same size

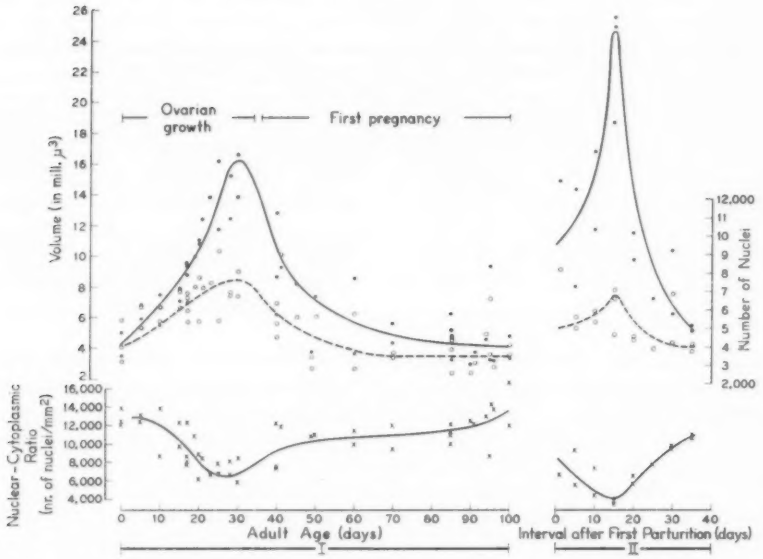

FIGURE 1. Graphic representation of morphological changes in the corpora allata of adult females of *Leucophaea maderae* in conjunction with reproductive cycles. ——●——●—— = volume of both corpora allata in million μ^3; ----○----○---- = number of nuclei, calculated for both glands; ——×——×—— = nuclear-cytoplasmic ratio (number of nuclei/mm.^{3}). Diagram includes (I) entire period of first reproductive cycle (from emergence of adult to first parturition at about 100 days of adult age), (II) second reproductive cycle (from first parturition to early part of second pregnancy). In this and subsequent figures of this series the curves do not represent mathematically correct summaries of the quantitative data which do not lend themselves to such treatment; they are merely intended to facilitate the visualization of the changes over the periods indicated. Note that volumetric changes are paralleled by changes in nuclear numbers and in nuclear-cytoplasmic ratios. For details see text.

as those of newly emerged females. This level was maintained throughout "pregnancy" to be followed by a more pronounced and more rapid rise (maximum volume 25.5 million μ^3) reached after 15 days in the pre-ovulation period of the second cycle. A study of this and older groups (Table I) indicated that essentially the same periodic changes in corpus allatum volume in conjunction with alternating phases of activity and inactivity occur in consecutive reproductive cycles. The first cycle differs from the subsequent ones only in degree in that the volumetric maximum is lower and is reached more slowly. In this respect our data agree with those of Engelmann (1957).

We came to different results, however, with respect to the remarkable cellular changes accompanying the periodic increase and decrease in corpus allatum volume. While it is true that the nuclei are more widely spaced in large, active corpora allata than in small, the rise in glandular volume is not exclusively due to an increase in the amount of cytoplasm. The contribution made by the nuclei in this growth process is primarily by a rise in their number, and less by an increase in

their size. In our series, the total number of nuclei estimated per pair of corpora allata rose from a minimum of 3128 in newly emerged females to a maximum of 9220 in the first, and of 8236 in the second pre-ovulation period.

The highest nuclear count of the whole series (15,591) belongs to a female which also has the highest volumetric value for normal corpora allata (37 million μ^3). This case is noteworthy in that it is a female with the exceptional adult age of 599 days carrying large ova at the time of fixation. Its corpora allata, in addition to the large number of normal sized nuclei, contain a giant nucleus (diameter 46 μ) comparable to those described by DeLerma (1932) and Palm (1947) in *Gryllotalpa*. The smallest number of nuclei (2782) occurred in small corpora allata of a 90-day-old female fixed before the onset of the second cycle. This shows the interesting fact that, with decreasing organ volume in pregnant females, the number of nuclei falls to reach again the level characteristic of very young adult specimens.

To sum up, during subsequent periods of activation and quiescence of the corpora allata the increase and decrease in organ volume is accompanied by periodic changes in nuclear numbers. The fact that the volumetric range (3.0–37.0 million μ^3 for both corpora allata) surpasses that of the nuclear numbers (2782–15,591) expresses itself in marked differences in nuclear distribution: Generally speaking, the larger the glands, the fewer the nuclei counted per mm.^{2} (range: 3698–16,765), and the higher the absolute and relative cytoplasmic content of the corpus allatum tissue. The cyclic fluctuations in nuclear numbers are so pronounced that cytological manifestations of these changes should be expected. Signs of mitotic activity, to account for the cell multiplication calculated, have been observed both in our normal and colchicine-treated adult specimens. The number of mitotic figures counted in the corpora allata is altogether small; while they appear to be more frequent during organ growth, mitoses are not entirely restricted to this period.

Conversely, signs of nuclear destruction (pycnosis) seem to be more pronounced in corpora allata returning to the inactive state, but are also occasionally found in growing or maximally active glands. This means that growth and regression of the corpora allata are not solely responsible for the fluctuations in nuclear numbers. The shifts in the frequency of mitotic and pycnotic nuclei can perhaps be better understood in conjunction with the cytological manifestations of the secretory activity of these glands.

While no comprehensive analysis of the elaboration of the secretory product by the corpora allata of *Leucophaea* is intended in this paper, certain statements may be made. Methods such as Gomori's chrome hematoxylin phloxine, Foot's modification of Masson's trichrome, or hematoxylin and eosin stain are not suitable for the demonstration of secretory materials in these glands. With the aldehyde fuchsin technique, distinct small granules can be demonstrated within the cytoplasm which stain from a rather deep purple to lavender. On occasion larger green staining droplets are observed. Newly emerged animals in our material do not show these granules; in older specimens their number and distribution appear to depend on the functional state.

During what seems to be a rather short and early phase in the secretory cycle certain corpus allatum cells stand out because their cytoplasm is densely packed

with stainable granules. Their presence permits the tracing of cellular processes which are the longer the more central the location of the cell within the gland. In other words, the corpus allatum cells in their mature form are stellate, and obviously release their secretory products by means of processes which end perpendicularly to the surface of the organ. This stellate cell shape cannot be readily ascertained in the absence of secretory granules, since cell boundaries are not always easily observed in the corpora allata. Many specimens show a more widespread distribution of granules which in sections can no longer be brought in spatial relationship with specific gland cells.

In cells presumably representing later stages in the secretory cycle the cytoplasm, instead of being homogeneous, assumes a more or less "stringy" appearance interspersed with vacuoles. These strands of cytoplasm form a characteristic pattern as though applied by strokes of a brush in a direction from the center to the periphery of the gland. It is along these "lines of flow" (Mendes, 1948; Özbas, 1957) that the secretory granules are now oriented, and their direction corresponds to that of the cell processes mentioned above. The fact that the granules tend to become lined up in greater numbers in the periphery of the corpora allata also speaks for their eventual release into the surrounding body fluid.

A further point of interest is that the nuclei of cells containing many secretory granules often appear pycnotic. These and additional pycnotic nuclei, not surrounded by secretory granules, range from slightly shrunken structures to homogeneous intensely staining bodies. Thus, there appears to be in adult corpora allata a continuous cellular turnover, whereby cells becoming exhausted in the process of their secretory function are replaced by the mitotic activity of younger probably non-secreting cell elements. The rate of cellular turnover seems to fluctuate in the course of a reproductive cycle, with the result that activation of the corpora allata is accompanied by increase, and return to inactivity by a decrease in cell numbers.

A further question concerns possible fluctuations in nuclear diameters in relation to periodic changes in organ volume. Measurements of representative cases have shown the nuclear diameter to vary only moderately in normal specimens (from 6.4 μ to 8.2 μ; mean 7.0 μ). The nuclei of any given specimen may fall within this range, and no definite relationship between organ volume and average nuclear size could be established. The measurements given do not include those of rarely found giant nuclei which seem to occur characteristically in old specimens (see above).

Of all the periodic changes in the appearance of the corpus allatum of *Leucophaea* the one most readily observed is that of the nuclear-cytoplasmic ratio. Thus, even without quantitative determinations, the large, *i.e.*, active corpus allatum can be easily distinguished from the inactive on the basis of its histological appearance.

b. Males

The 46 male specimens studied range from an adult age of 0 to 471 days (Fig. 2). As in the female series, the corpora allata of animals of the same age may show a certain variability in size and nuclear distribution. The smaller average body size of males is reflected in lower corpus allatum values. Shortly after

emergence the glands begin to grow, but this period is shorter than in females. After about 10 days a peak is reached which, on the average, amounts to a volumetric increase of 2½ times over the initial volume. After this, the values level off to fluctuate only mildly throughout the adult stage. The volume of the largest pair of glands measured in this series (9.0 million μ^3) is about four times that of the smallest (2.1 million μ^3). As one might expect, in analogy to the situation in females, the variability in total nuclear counts is somewhat lower in degree than that in volume, but it is nevertheless considerable (range: 2032 to 7000).

Thus, if arranged in the order of increasing volume, male corpora allata also show a gradual increase in the relative amount of cytoplasm. The number of nuclei per mm.^{2} ranges from 7696 to 17,131. Inasmuch as male corpora allata are on the average smaller than female, their nuclei are generally more crowded. The highest density occurred in a case (adult age: 51 days) which also had the largest absolute number of nuclei; the corpora allata were of medium size. The loosest arrangement of nuclei was observed in an old specimen (adult age: 396 days) with large corpora allata (7.4 million μ^3). The relative cytoplasmic content was almost as high in a male (adult age: 10 days) which had the largest corpora allata in the entire series.

Figure 2 shows quite clearly that the rise in corpus allatum volume during the first ten days of adult life is accompanied by an increase in nuclear numbers and a decrease in the number of nuclei per unit of tissue. The reverse trend occurs after the peak, *i.e.*, in males older than 10 days, but as a group these males do not entirely return to the situation characteristic of newly emerged animals. In principle, the male corpora allata show the same relationships between organ size, number of nuclei, and density of nuclear arrangement as the females, but this relationship is not so pronounced. It is interesting, for example, that within a medium size range (4–5 million μ^3) which encompasses the majority of males

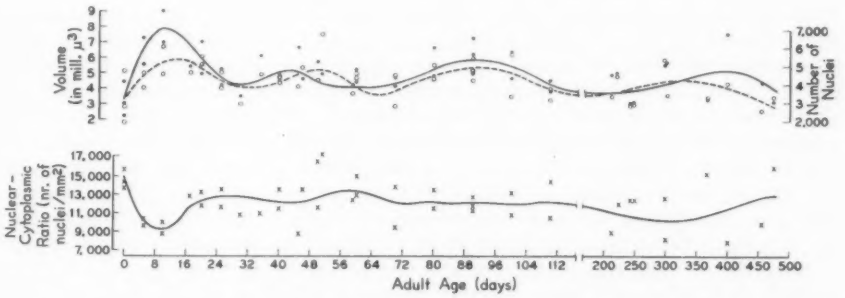

FIGURE 2. Diagram showing morphological characteristics of corpora allata of males of *Leucophaea maderae* ranging in adult age from 0 to 471 days. After a small initial rise (maximum at 10 days) the volumes (——●——●——) level off to fluctuate around a mean value maintained throughout the adult life span. A lack of distinct cyclic activity is also apparent from the values expressing nuclear numbers (-----○-----○-----) and nuclear-cytoplasmic ratios (——×——×——).

studied, the nuclear-cytoplasmic ratio shows the highest degree of variability as compared to that in groups with smaller or larger glands.

The data mentioned so far do not provide evidence for the existence of cyclic activity changes in the corpora allata of adult males. This also applies to the cytology of secretory processes. Purple-staining granules in the cytoplasm have been found in varying amounts in animals of different ages with the exception of newly emerged specimens. While indicating the existence of a secretory function in male adult corpora allata, these cytological phenomena are less impressive than those in females which they resemble in principle. The fact that signs of secretory cycles as well as volumetric and nuclear changes are less apparent in males is related to the greater range in the response of female corpora allata to varying physiological conditions.

Discussion

In most species of insects studied, extirpation and implantation experiments have established the control of reproductive processes by a hormone of the corpora allata (for review see Scharrer, 1955). There are also numerous indications that, at the height of their "gonadotropic" activity, the corpora allata of females are larger than when they are inactive. This was reported among others by Ito (1918), Wigglesworth (1936), Thomsen (1942), Palm (1947), Mendes (1948) and Kaiser (1949). However, most of these and more recent studies (Müssbichler, 1952; Nayar, 1956; DeWilde, 1954; Brandenburg, 1956; Lukoschus, 1956; Lhoste, 1957) do not extend beyond a relatively short interval between emergence and oviposition. A more detailed analysis of this relationship was carried out by Engelmann (1957) who correlated corpus allatum volume and structure with ovarian activity in *Leucophaea maderae,* the species which was also used in the present study. During the long life span of this "ovo-viviparous" species, periods of ovarian quiescence during "pregnancy" alternate with those of activity in which the terminal oocytes grow and deposit yolk, and the accessory sex glands produce secretory material. Only this active phase in the reproductive cycle requires the presence of the corpus allatum hormone while the maturation of the embryos proceeds without it (Scharrer, 1946b). When Engelmann found large corpora allata with relatively high cytoplasmic content in females approaching ovulation, and small glands with densely packed nuclei during pregnancy, he concluded that "activation" of the corpora allata is characterized by an increase in the amount of cytoplasm while the number of nuclei remains constant. The present study, based on a larger material, confirms these earlier results as far as the periodic changes in corpus allatum volume are concerned; it also offers evidence that these changes continue beyond the period (135 days after emergence) analyzed in Engelmann's study, *i.e.*, throughout the adult (reproductive) phase of the insect. In addition, our results demonstrate a participation of the nuclei in the activity cycles of the corpora allata. The role of the nuclei could be ascertained only by quantitative methods in a sufficiently large material, since cell divisions are not observed frequently enough in normal adult specimens. Nuclear counts have shown that, in the first as well as subsequent reproductive cycles, an up to four-fold increase in the number of nuclei may take place during the phase of growth and activation of the corpora allata. The decrease in organ volume which follows is accompanied by a corresponding decrease in nuclear number. These differences in total nuclear

counts are so large that they allow a considerable margin of error which cannot be avoided in the calculation of these figures.

While our cytological observations do not lend themselves to a quantitative evaluation of nuclear changes during the activity cycles of the corpora allata, it is significant that signs of mitotic activity are more prominent in growing glands and pycnotic nuclei are more conspicuous in corpora allata returning to the inactive state. These observations in correlation with the study of the cytology of the secretory cycle lead to the conclusion that corpus allatum cells are used up and replaced during the adult life of the animal. Cyclic changes in the rate of this cellular turnover which accompany secretory cycles in these glands account for the periodic increase and decrease in nuclear numbers and accompanying fluctuations in organ volume.

By comparison, nuclear size showed less variability in our material, so that the main contribution of the nuclei toward increase in organ volume is due to a rise in their number. Taken as a whole, the participation of the nuclear component stays behind that of the cytoplasm which shows not only pronounced quantitative changes but undergoes qualitative transformations during the process of elaboration of secretory granules.

The use of the aldehyde fuchsin technique in the present study for the first time permitted the demonstration of secretion granules in the corpora allata of *Leucophaea*. Although details of the whole secretory cycle still need to be worked out, present evidence supports the view that the active principle is elaborated in the cytoplasm of stellate cells and released into the body fluid surrounding the surface of the gland. Depending on the more peripheral or central location of a cell, its processes may be short or long. They account for the characteristic structural pattern of the gland during certain stages of the cycle in which strands of cytoplasm directed toward the periphery and interspersed with vacuoles are delineated by rows of secretory granules which become more numerous in the periphery of the gland. It is uncertain whether a gland cell remains active only during one secretory cycle or not, but the frequent observations of more or less pycnotic nuclei in cells filled with the secretory product indicate that the cells may become exhausted and are replaced by cells resulting from mitotic divisions in the adult gland. The observation of cytoplasm becoming vacuolized during phases of activity as well as the arrangement of secretory granules along "lines of flow" (determined by the peripheral direction of the cell processes) is in agreement with the findings of other authors in different species of insects (Mendes, 1948; Özbas, 1957).

Thus, in the cyclic activity of the corpora allata of adult females of *Leucophaea* a number of factors are involved. The question arises which of the changes observed express the physiological activation of the corpus allatum. This question cannot be fully answered as yet, but certain known data are of interest in this connection. Increase in nuclear number without relative cytoplasmic increase can be achieved in adult female glands by the implantation of prothoracic glands (Engelmann, personal communication). The absence of ovarian stimulation in animals thus treated indicates that in the corpus allatum of adult *Leucophaea* the relative cytoplasmic increase is an important prerequisite for its activation. The possibility that it is the only one is illustrated by the situation in those species where corpus allatum growth in adults is said to take place solely by an increase in cell volume and

nuclear volume, but not in cell number (Brandenburg, 1956; Lukoschus, 1956). It is evident from Engelmann's data as well as our own (Fig. 1) that at the first peak of activity the corpus allatum volume does not reach the same level as at the second and subsequent ones. Conversely, more time is required for the stimulation of the ovary during the first reproductive cycle than later on. It seems that the corpus allatum may require a short period after emergence to complete its adult development, as has been postulated also for other organs (Rockstein, 1956). The gland may, therefore, not be capable of complete "activation" until after the first cycle.

The possibility that such post-emergence maturation takes place is also indicated by the moderate rise in volume and nuclear numbers occurring in male corpora allata within the first ten days of adult life. Our own observations in this respect are in line with those of Engelmann (personal communication) in *Leucophaea* and by Mendes (1948) in *Melanoplus*. At any rate, no interpretation other than one of tissue maturation can be given for the initial volumetric increase of the corpora allata of adult males, as long as their functional role is so little understood. All we know is that the male reproductive activity in *Leucophaea* is undisturbed after allatectomy (Scharrer, 1946b), and that certain data suggest a relationship between corpora allata and metabolic processes (Samuels, 1956). A sustained control of metabolic functions by the corpora allata might well account for the picture of "mild activity" frequently observed in histological preparations of adult male glands of every age. This steady appearance, which is in contrast to the cyclic pattern in the corpora allata of adult females, concerns range in organ volume and nuclear number as well as cytological manifestations of secretory activity.

In connection with the observation in both sexes of corpus allatum stimulation following emergence it is of interest that this early adult period in *Leucophaea* also differs from later ones with respect to certain metabolic data, such as the animal's lipid content (Scharrer and Wilson, unpublished data).

The present study illustrates that the histophysiological approach whose value is well recognized in vertebrate endocrinology is equally fruitful in the exploration of endocrine mechanisms in insects. In the special case of the corpora allata of *Leucophaea*, known variations of their activity are paralleled by marked changes in the volume of the entire organ, the number of cells, the nuclear-cytoplasmic ratio, and the cytology of the secretory process. The subsequent papers will be concerned with corpus allatum structure following experimentally induced changes in the normal pattern of activity and quiescence of this gland.

Summary

1. The corpora allata of *Leucophaea maderae* display a remarkable degree of structural variability in conjunction with changing functional states. This is particularly apparent in adult females, where a regular sequence of activity and inactivity of these glands parallels alternating phases of ovarian development and quiescence.

2. The volume of active corpora allata surpasses that of inactive glands beyond the range of individual variation, which is considerable. The volumetric rise signalling activation is accomplished to a large extent by an absolute and relative

increase in cytoplasmic content which results in a characteristic "loose" distribution of the nuclei.

3. The present study shows further that the nuclei participate significantly in the cyclic changes of these organs. During each growth period, the nuclear numbers increase up to several times the original values. When, after ovulation, the corpus allatum returns to a state of inactivity which is maintained during pregnancy, the nuclear-cytoplasmic ratio returns to a level characteristic of the newly emerged female. The accompanying reduction in cell number to the initial level is evident not only from a drop in nuclear counts but from the observation of pycnotic nuclei.

4. The differences in the frequencies with which nuclear pycnosis on one hand, and mitotic figures on the other are observed in various stages suggest the existence of a cellular turnover which seems continuous but whose rate changes periodically. During activation of the corpus allatum, when secretory products are elaborated, the increase in cell number surpasses the rate of cell destruction. In the regressing gland the latter process predominates over that of cell replacement.

5. With the use of a modified aldehyde fuchsin technique distinct secretory granules have been demonstrated in the corpus allatum cells of *Leucophaea*. The granules line up along processes of the cells which are directed to the surface of the corpus allatum. This fact, as well as the accumulation of stainable granules in the periphery of the gland, speaks for the release of the active substance into the surrounding hemolymph. The occurrence of pycnotic nuclei in cells filled with secretion granules suggests that these gland cells may become exhausted fairly quickly, perhaps in the course of one secretory cycle.

6. By comparison with the situation in the females, the corpora allata of adult males show considerably less variability. Soon after emergence, a short period of "activation" seems to occur, as judged by the same structural characteristics as in the females. After that a fairly constant level of presumably mild activity appears to be maintained throughout adult life. Since the available information on the functional role of the corpora allata in male adult animals suggests no pattern of periodicity, the lack of distinct cyclic changes in the morphology of these glands is not surprising.

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HISTOPHYSIOLOGICAL STUDIES ON THE CORPUS ALLATUM OF LEUCOPHAEA MADERAE. II. THE EFFECT OF STARVATION[1]

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The corpora allata of *Leucophaea maderae* undergo marked histological changes in conjunction with various phases of the reproductive cycle (Engelmann, 1957; Scharrer and von Harnack, 1958). Activation of the corpus allatum is characterized by an increase in organ volume, in nuclear number, and in absolute and relative cytoplasmic content. These changes, as well as the cytological manifestations of secretory activity, reach a peak shortly before ovulation. During the subsequent period of pregnancy, when the ovaries presumably receive no hormonal stimulation, the corpora allata return to the inactive condition (small size, dense nuclear arrangement) in which they remain until the onset of the next reproductive cycle.

The decision as to whether or not the corpora allata become activated at any given time resides in the central nervous system which, under certain conditions, exerts a restraining influence on these glands. In addition, nervous or neurohumoral stimuli appear to be necessary to sustain the activity of the corpora allata (Scharrer, 1952; Engelmann, 1957). The type of message sent to the corpora allata is determined by a variety of afferent impulses from the external and internal milieu (see Scharrer, 1958, 1959). One of these is the nutritional state of the animal.

During a period of total starvation egg development is suppressed in *Leucophaea* (Scharrer, 1946); however, the ovary remains capable of responding to implanted active corpora allata (Johansson, 1955). From this result one can conclude that the absence of nutrients acts as a stimulus to the brain eliciting an inhibitory message to the corpora allata.

It was of interest, therefore, to examine the effects of inanition on the morphology of the corpora allata of adult females of *Leucophaea maderae* (von Harnack, 1958). The present study is concerned with two aspects of this problem: (1) Does prolonged total starvation affect the structure of the corpora allata, either to or perhaps beyond the point of preventing their activation? (2) How do corpora allata, kept inactive by starvation for a considerable period, respond to the resumption of a normal diet?

Material and Methods

The material on which this investigation is based consists of two series (A and B), one in which the animals were subjected to total starvation for various periods

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[2] Grantee of the American Association of University Women.

of time, and another in which a period of starvation was followed by return to a normal diet. All animals used were adult females removed from the stock colonies on the day of emergence and isolated in pint-size jars. At the same time the period of starvation was initiated during which the majority of animals received only water; a small number was deprived of water as well as food.

Series A was used for the study of the influence of prolonged total starvation on the morphology and function of the corpora allata. It consisted of 52 females on a water diet, and of 31 females starved and dehydrated at the same time. The animals were fixed at intervals ranging from 5 to 95 days following the beginning of the starvation period, *i.e.*, the specimens surviving longest had reached an adult age at which normally the first reproductive cycle would have been completed. Animals showing increasing signs of weakness before being fixed could be presumed to have largely exhausted their nutritional resources. As might be expected, the point of exhaustion was reached sooner in the dehydrated group.

Series B gives information on the response of the corpora allata to the resumption of a normal food intake following an extended starvation period. In this group 51 newly emerged females received nothing but water for 30 days. They were then returned to a regular diet of dog chow and apple; they were kept together with normal males, and allowed to survive for up to an additional 90 days. In both experimental series, fixation was scheduled at five-day intervals, except during periods when more pronounced structural changes of the corpora allata were observed and, therefore, one- or two-day intervals became desirable.

Autopsies were performed in all cases in order to ascertain the condition of the reproductive organs. The histological procedure, and the method of quantitative evaluation of the corpora allata were the same as reported in the preceding paper of this series (Scharrer and von Harnack, 1958).

Results

Series A: Starved animals

In contrast to the corpora allata of normally fed females which reach a four-fold increase in volume within 30 days after emergence, the corpora allata of starved females are noticeably suppressed (Fig. 1 and Fig. 3). The situation in the group of 52 animals kept on a water diet was as follows. During an initial period of 15 days, when inanition had not yet become effective, the corpora allata grew at a rate comparable to that in normally fed animals, *i.e.*, their volume doubled. After that the corpus allatum volume of starved females showed a gradual but continuous decrease. At 30 days of adult age the corpora allata had returned to the level of the newly emerged female, and the longest survivor, fixed after 95 days, had reached a minimal corpus allatum volume of 1.7 million μ^3. This means that prolonged starvation had caused a decrease of corpus allatum volume to about one-half of the minimal size found in normally fed adult females.

The initial rise in corpus allatum volume of the starvation series is paralleled by an increase in nuclear numbers which compares with that in the normal control series. The turning point at which the nuclei begin to decrease in number occurs sooner in the experimental group (Fig. 1) than in the controls. Subsequently the nuclear counts return to a value characteristic of the normal inactive gland, while the cytoplasmic content drops considerably below the normal baseline. The

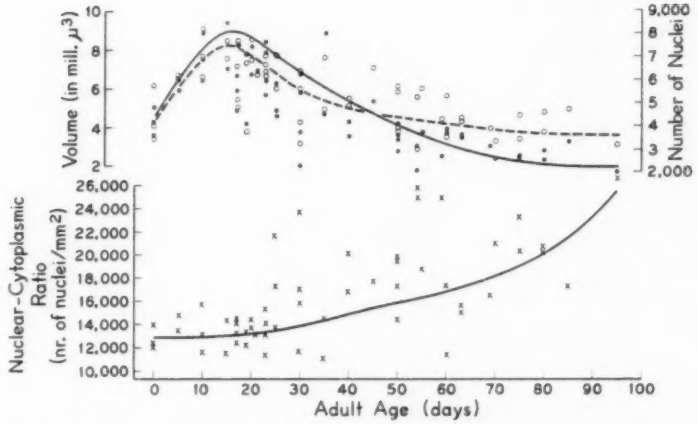

FIGURE 1. Diagram indicating morphological changes in the corpora allata of adult females of *Leucophaea maderae* which received only water from the day of emergence. Note that the volume of the corpora allata (——●——●——) and their number of nuclei (-----○-----○-----) fall after a moderate initial rise. ——×——×—— = nuclear-cytoplasmic ratio. For comparison with normal controls see Figure 3.

result is a very crowded nuclear arrangement in the corpora allata of specimens fixed after excessively long periods of starvation.

These stepwise histological changes are enhanced in the starved-dehydated group. When combined with dehydration, the first manifestations of inanition become evident earlier; the turning point in corpus allatum volume occurs already at about 12 days of adult age and appears accordingly somewhat lower on the curve (Fig. 3) than that in the starved-hydrated group. Thus the decline in corpus allatum volume which follows the moderate peak in the dehydrated group runs roughly parallel to that in the hydrated group and reaches the endpoint sooner (Fig. 3). Another indication of the aggravating effect of dehydration on starving animals was the extreme crowding of nuclei which made quantitative estimates of reasonable accuracy impossible. Nuclear counts were, therefore, omitted in the starved-dehydrated group of animals. In the present as well as earlier experimental series survival rates were consistently lower in starved-dehydrated than in starved-hydrated groups. The longest survival recorded in the dehydrated group was only 65 days.

As was to be expected from preceding studies (Scharrer, 1946; Johansson, 1955), none of the females deprived of food from the day of their emergence showed signs of reproductive activity. On autopsy, only small undeveloped eggs were observed in the ovaries, and the accessory sex glands contained no appreciable amount of secretory material.

Series B: Starved and re-fed animals

In this group of females the return to a normal diet occurred after a starvation period of 30 days in which only water was available; *i.e.*, at a time when the

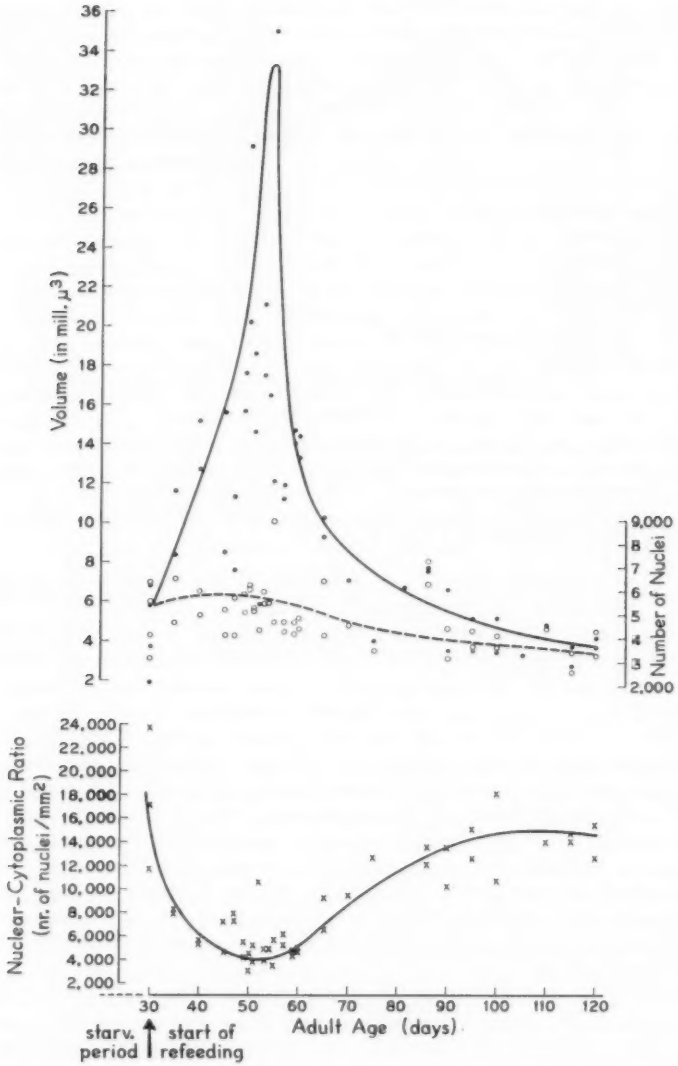

FIGURE 2. Diagram showing the response of the corpora allata in adult females of *Leucophaea maderae* which were starved, with access to water, for 30 days following emergence and were then returned to a normal diet. ——●——●—— = volume of both corpora allata; -----○-----○----- = number of nuclei of both corpora allata; ——×——×—— = nuclear-cytoplasmic ratio (number of nuclei/mm²). Compare volumetric changes with those of normal controls (Fig. 4).

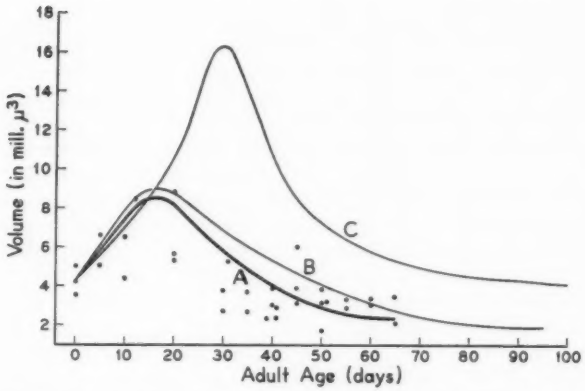

FIGURE 3. Diagram illustrating changes in the volume of both corpora allata of females of *Leucophaea maderae* which were starved and dehydrated following emergence (curve A). Compare with group of starved-hydrated animals (curve B) and with normally fed control group (curve C).

corpus allatum volume as well as the nuclear counts had almost returned to baseline levels characteristic of the normal inactive gland. Re-feeding resulted in a rather dramatic response of the corpora allata. Within 15–20 days, a peak was reached which represented a seven-fold increase in volume (Fig. 2). This considerably surpasses the peak characteristic of the growth curve of the first reproductive cycle under normal feeding conditions (four-fold increase in volume). It compares favorably with that of the second normal cycle which it also resembles with respect to the steepness of the slopes (Fig. 4). It appears as though the starved animal, on resumption of feeding, is capable of "making up for lost time" and does not have to start its reproductive period as "gradually" as the normal animal.

The rapid rise of corpus allatum volume in the starved and re-fed series is not accompanied by a correspondingly high increase in nuclear number (Fig. 2). Consequently, the largest corpora allata have an exceptionally high content in cytoplasm, and accordingly the nuclei are more widely spaced than in the most active glands of the first reproductive cycle of the normal series.

The full-scale activation of the corpora allata in this first reproductive cycle of the starved-re-fed animals promptly led to stimulation of the ovaries; ovulation occurred within about 25 days after the resumption of feeding.

DISCUSSION

In the present as well as earlier experiments (Scharrer, 1946; Johansson, 1955; Willis and Lewis, 1957), the roach *Leucophaea maderae,* when subjected to total starvation, showed a considerable capacity to survive. If water was provided, adult females which were starved from the day they emerged lived for up to three months at room temperature. This period was shortened by about three weeks, when the insects were deprived of water as well as solid food. In *Leucophaea maderae,* as in certain other insect species, eggs do not develop in the total

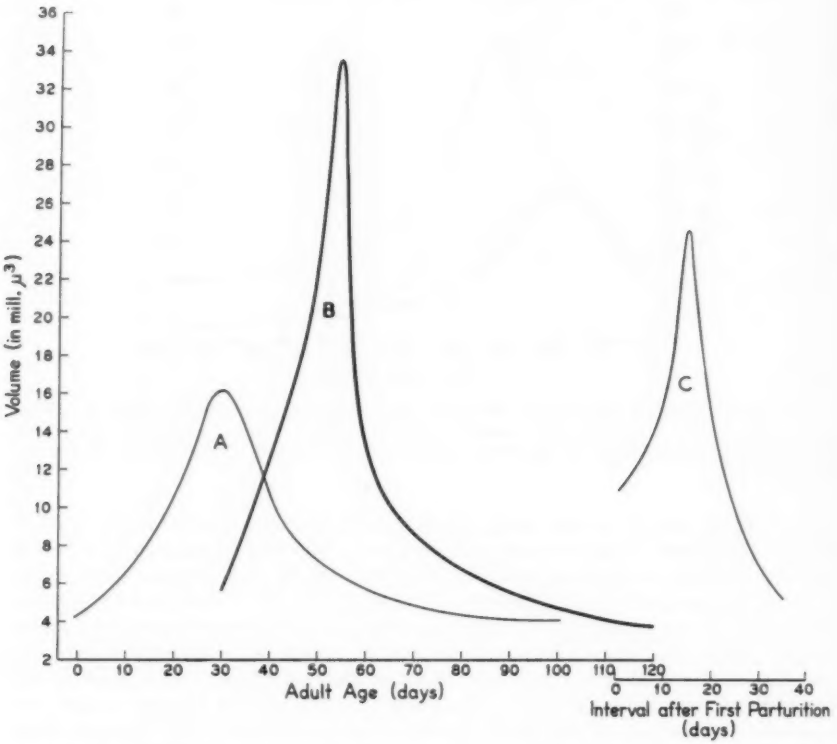

FIGURE 4. Diagram showing the pronounced rise in corpus allatum volume of starved-re-fed females (curve B), in comparison with corpus allatum growth in first normal (curve A) and second normal reproductive cycle (curve C).

absence of nutrients (Scharrer, 1946), unless active corpora allata are implanted (Johansson, 1954; 1955), or the starving insect's own corpora allata are released by surgery from the action of the restraining nerves (Johansson, personal communication). These results demonstrate that (a) the gonads of starved animals have the capacity to respond to gonadotropic stimulation, and (b) the initial moderate rise in corpus allatum volume observed in starving *Leucophaea* females seems to represent a degree of activation insufficient to elicit ovarian response.

The longer the period of inanition, the smaller and the poorer in cytoplasmic content are the corpora allata. It is difficult to estimate the number of nuclei present in these small organs, and to demonstrate possible signs of secretory activity. At the endpoint, beyond which survival was no longer possible, the corpora allata had only about one-half the volume of normal female "inactive" glands. It cannot be determined with certainty whether or not this decrease below the normal range of corpus allatum size is nothing more than a general effect

of inanition shared by other organs of the body. An attempt was made to measure organs in the vicinity of the corpora allata, such as the musculature of the head. A comparison of the diameters of muscle fibers in normal and starved specimens suggests that some "wasting" occurs in the latter. This is also evident from the larger spaces between muscle fibers in drastically starved animals. However, since the individual muscle elements vary in width, a reasonably accurate estimation of the degree of shrinkage is not possible.

What was said so far applies to starved animals receiving water, and to an even larger degree to starved-dehydrated specimens. Their corpora allata do not even reach the size of the starved-hydrated group, and the decline in volume (and activity) occurs proportionately sooner. A comparable dependency of the morphology and function of the corpus allatum on the nutritional state has been observed also in several other insect species (Wigglesworth, 1936; Schwinck, 1951; Müssbichler, 1952; Engelmann, 1957).

In a general way, the effects of nutritional deficiency on the corpus allatum of the insects compare with those on its analogue, the anterior pituitary of the vertebrates.

A variety of studies in mammals have shown that, under conditions of starvation, (a) distinct structural and functional changes occur in the pars anterior, (b) gonadal malfunction is attributable to suppression of gonadotropic activity, and (c) the reproductive system deficient because of starvation responds to the administration of pituitary material.

The morphological changes observed in pituitaries of starving laboratory mammals as well as human patients, such as decrease in the volume or weight of the anterior lobe (Jackson, 1917), in the size and number of parenchymal (acidophilic) cells (Sedlezky, 1924; Schubothe, 1940; D'Angelo *et al.*, 1948), and in relative cytoplasmic content (Jackson, 1917), are in line with the changes described in the present study for the corpora allata of the insect, *Leucophaea*.

As in the insect, the concomitant disturbance of gonadotropic activity in the mammals studied led to depression of gonadal function (Mulinos and Pomerantz, 1940; Rinaldini, 1949/50) which could be remedied by the administration of gonad-stimulating substances (Boutwell *et al.*, 1948; Rinaldini, 1949/50).

It was of particular interest to observe the effects of the return to a normal diet in females of *Leucophaea* whose ovarian activity had been restrained by a 30-day starvation period following emergence. Instead of beginning their reproductive period in the same manner as young normal adults, even though belatedly, starved-re-fed females "skipped the first cycle" for which a slower and more moderate activation of the corpora allata is typical. At once they acted at full capacity. Thus, the return to ample food supplies may represent a powerful stimulus for corpus allatum growth. Within a short period of 20–25 days an up to seven-fold volumetric increase occurred. The subsequent decline from these high values was equally rapid. From every point of view, the curves illustrating this reproductive cycle do not differ essentially from those characteristic of the second or subsequent cycles in normal specimens (Figs. 2, 4). In fact, the peak illustrating the range of corpus allatum activation is higher in the starved-fed group than in any cycle of our normal series. However, this difference might perhaps be due to individual variation, since Engelmann's (1957) values for the second reproductive cycle of

normal females reach approximately the same maximum as that obtained in the present series of experimental animals. On the other hand, the values obtained by Engelmann are not entirely comparable to those reported here, because his experiments were conducted under different conditions of temperature and humidity. At any rate, there can be no doubt that corpora allata of animals having been starved for some time and then returned to normal food supply, respond with great readiness and display pronounced signs of activation. The same observation was made in nymphs of *Panorpa* (Schwinck, 1951). In contrast to a newly emerged animal, a starved-fed female undergoing its first reproductive cycle has had time to adjust to the changes connected with "metamorphosis." This may be the reason why its corpora allata, like those of older normal specimens, respond maximally as soon as the restraining effect of the brain is lifted. This would mean that the "post-emergence maturation" postulated by Rockstein (1956) can take place under conditions of total starvation.

Be this as it may, within certain limits the degree of corpus allatum activation obtained under various normal and experimental conditions is perhaps not too significant. One must keep in mind that the more moderate activation of the corpora allata in the first normal cycle suffices for the development of a full set of eggs. The more pronounced response of the corpora allata in more mature adults, normal as well as starved-fed may be the result of a higher metabolic rate of their tissues. This possibility is suggested by comparable data in mammals. Here, re-feeding after starvation resulted in an increased metabolic rate (Quimby *et al.*, 1948) and in a prompt response of the anterior lobe (Jackson, 1917).

Summary

1. Adult females of *Leucophaea maderae* were subjected to total starvation, with or without dehydration, following their emergence. Throughout the period of survival, *i.e.*, up to 95 days, no egg development occurred as a consequence of the failure of the corpora allata to become properly activated. The corpora allata of a series of starved animals fixed after varying intervals showed only a small initial rise in volume which was followed by a gradual decrease reaching a minimum below that of normal controls.

2. In another series of animals, starved for 30 days, the return to a normal diet promptly initiated a growth phase in the corpora allata which considerably surpassed that characteristic of the first reproductive cycle in normal animals (seven-fold, instead of four-fold volumetric increase). As to speed and degree, this period of activation compared favorably with that of the second cycle in normal females. Thus the delay in reproductive activity, caused by starvation, was at least in part compensated for by a more rapid and complete activation of the corpora allata which in turn promptly led to ovarian development.

3. The effects of starvation and re-feeding on the structure and function of the corpora allata of *Leucophaea* are in line with those described in the literature for the analogous organ in mammals, *i.e.*, the anterior lobe of the pituitary.

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SUBSTANCES WITH JUVENILE HORMONE ACTIVITY IN CRUSTACEA AND OTHER INVERTEBRATES[1]

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The cyclical growth and molting of immature insects is brought about by two hormones, one secreted by the insect's brain and the other by the prothoracic glands. A third hormone, the juvenile hormone, is secreted by the corpora allata, endocrine glands in the head or prothorax of the insect. This hormone promotes larval development but prevents metamorphosis (Wigglesworth, 1957). Its presence in the immature insect guarantees that when the larva molts it will retain its juvenile characters and not differentiate into an adult. The juvenile hormone is thus a remarkable molecule that permits growth but prevents maturation. So far as we are aware it has no functional counterpart in the vertebrates. Recently Williams (1956) has reported that ether extracts of the abdomens of male Cecropia moths (*Hyalophora cecropia* L.) contain large amounts of juvenile hormone. When this extract was injected into lepidopterous pupae, they molted into second pupae instead of molting into adults. This, of course, is precisely what occurs when active corpora allata are implanted into pupae (Piepho, 1951; Williams, 1952).

Although initial experiments demonstrated juvenile hormone only in extracts of male Cecropia moths, we have since extracted it from both males and females of 22 species of Lepidoptera representing 6 families (Schneiderman and Gilbert, 1957; Gilbert and Schneiderman, 1958a). This result suggested that the hormone could have a wider distribution in the animal kingdom. The experiments to be reported were conducted to determine whether substances with juvenile hormone activity could be extracted from other insect orders besides Lepidoptera, from other classes of arthropods and from other phyla.

Materials and Methods

1. *Experimental animals*

Pupae of the polyphemus silkworm (*Antheraea polyphemus* Cram.) were used as test-objects for assay of juvenile hormone activity. They were stored for about thirty weeks at 6° C. prior to use.

2. *Preparation and assay of extracts*

Animals representing most of the major groups of invertebrates were collected at Woods Hole, preserved in methanol and shipped to Cornell University for ex-

[1] This investigation was supported by grant H-1887 from the National Heart Institute of the U. S. Public Health Service.

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traction. Some animals (*e.g.*, earthworms and slugs) were collected locally and extracted immediately. The tissues were homogenized in ethyl ether and the homogenates and methanolic extracts vigorously re-extracted with ether in a continuous extractor. The ether extracts were washed several times with water, the ether evaporated off and the oily or waxy residue dried *in vacuo* at 60° C.

To test for juvenile hormone activity in the resulting extracts, many of which were toxic and waxy, a new and exceedingly sensitive assay procedure was developed which permitted detection of traces of juvenile hormone activity in crude extracts. The assay takes advantage of the extraordinary sensitivity of regenerating epidermal tissue to juvenile hormone (Piepho, 1950; Piepho and Heims, 1952). The extract to be assayed is mixed with peanut oil and paraffin wax. A small rectangle of integument is excised from the thorax of a Polyphemus pupa, a few crystals of streptomycin and phenylthiourea (an anti-tyrosinase) placed in the wound, and the wound sealed with a few milligrams of melted wax-peanut oil-extract mixture. When the adult moth emerges three to four weeks later, the wound area is examined. In the case of inactive extracts, the only evidence of the former wound is a small indentation covered with adult cuticle. However, if the extract is active, then an island of pupal cuticle occurs at the wound site. Figure 1 depicts such a patch of pupal cuticle. It stands out sharply from the adult cuticle which surrounds it. It is scale-less, brown, rugose and typically pupal in

FIGURE 1. Thorax of adult Polyphemus with a pupal patch produced by the wax test. Scales have been removed.

TABLE I

Effects of serial dilutions of crude juvenile hormone extract in paraffin

Concentration of hormone in paraffin	Effect*		
0 (Peanut oil)	0	0	0
0 (Inactive oils)	0	0	0
0 (Paraffin)	0	0	0
1:2000	+	0	0
1:512	+	0	0
1:256	+	0	0
1:128	+	+	0
1:64	+	+	0
1:32	+	+	+
1:16	+	+	+
1:8	+	+	+
1:4	+	+	+
1:1	+	+	+

* Each symbol represents a test animal.

most other respects; it may even have pupal setae. In cross-section it appears to be three to four times as thick as adult thoracic cuticle. In short, it is essentially indistinguishable from ordinary pupal cuticle.

This "wax test" appears to be far more sensitive than other tests for juvenile hormone activity, as the following experiment reveals. A crude ether extract of the abdomens of male Cecropia moths was serially diluted with peanut oil (up to 1/1000) and these dilutions dissolved in equal parts of wax and applied to thoracic wounds as described above. The results recorded in Table I reveal that the assay permits detection of final dilutions of hormone of 1/2000. That is, in principle, an extract that contained only 1/1000th as much juvenile hormone activity as male Cecropia extract would yield a positive result. In the 1/2000 dilution recorded in Table I, the wax patch weighed about 6 milligrams and, therefore, contained only *3 micrograms* of crude extract. Hence it is possible with this test to assay the juvenile hormone content of minute quantities of material extracted from a part of a single insect.

It is important to note that, so far as we can ascertain, the wax test is absolutely specific for juvenile hormone activity. Thus wax alone, or mixtures of wax with peanut oil, have never given us false "positive tests" although dozens of control tests have been made. Moreover, when the active principle is removed from the crude extract by repeated liquid-liquid extractions, the oil that remains, containing virtually all of the ether-extractable material in the original extract, is also inactive in the wax test.

RESULTS AND DISCUSSION

Using this sensitive test, extracts from 13 classes of invertebrates representing most of the major phyla were examined. The results presented in Table II reveal that ether extracts of a truly diverse array of invertebrates possess at least some juvenile hormone activity. It is not too surprising to find juvenile hormone activity in crustaceans and even in annelids, but surely its presence in hydroids and sea cucumbers is unexpected.

TABLE II

Juvenile hormone activity of ether extracts of various invertebrates

Phylum	Class	Species	Wax test
Porifera	Demospongiae	*Microciona prolifera*	0
		Cliona celata	0
Cnidaria	Hydrozoa	*Pennaria tiarella*	+
		Tubularia crocea	+
	Anthozoa	*Metridium dianthus*	0
Rhynchocoela	Anopla	*Cerebratulus* sp.	
		(bodies)	0
		(heads)	0
Annelida	Polychaeta	*Nereis virens*	
		(bodies)	+
		(heads)	+
	Oligochaeta	*Lumbricus terrestris*	
		(bodies)	+
		(heads)	+
Arthropoda	Insecta	Numerous Lepidoptera	+
		Tenebrio molitor (Coleoptera)	
		(larvae)*	+
		(adults)	0
		Sarcophaga bullata (Diptera)	
		(larvae)	0
		Neodiprion lecontei (Hymenoptera)	
		(diapausing prepupae)	0
		Apis mellifera (Hymenoptera)	
		(winter workers)	0
	Crustacea	*Uca pugilator*	+
	(Decapoda)	*Orconectes immunis*	
		(entire)	0
		(purified extract)	0
		Homarus americanus	
		(eyestalks)	+
		Carcinides maenas	
		(fronts)	0
		(rears)	0
		Palaemonetes vulgaris	0
	Arachnida	*Limulus polyphemus*	
		(fronts)	0
		(rears)	0
		(purified sterols)	0
Mollusca	Gastropoda	*Deroceras* (*Agriolimax*) *agreste*	
		(heads)	0
Echinodermata	Holothuroidea	*Thyone briareus*	0
		Leptosynapta inhaerens	+
	Echinoidea	*Arbacia punctulata*	0
Enteropneusta	Balanoglossida	*Saccoglossus kowalevskii*	
		(entire)	+
		(less collar and proboscis)	+

* Tested by injecting extract.

It is of some interest that the most potent non-insect extract came from the eyestalks of lobsters. The occurrence of high concentrations of substances with juvenile hormone activity in the eyestalk, which is a well-known endocrine center

in crustaceans (Knowles and Carlisle, 1956), suggests that in crustaceans the eyestalk may contain a gland which produces a substance chemically similar to the juvenile hormone of the corpora allata. A likely site is a part of the X-organ which is not neurosecretory but appears glandular (*i.e.*, the secretory cells of the sensory papilla X-organ (Knowles and Carlisle, 1956)). Whether the juvenile hormone plays a role in crustacean development or egg maturation remains to be proved, but it appears likely. In addition to these results we have also recently found juvenile hormone activity in the adrenal cortex of cattle (Gilbert and Schneiderman, 1958b). Hence, it seems safe to conclude that substances with juvenile hormone activity are widespread in the animal kingdom. As far as we are aware, the only other animal growth hormones of such wide distribution are the estrogens (Loewe *et al.*, 1932; Hagerman *et al.*, 1957).

Whether or not these juvenile hormone substances are similar chemically to the juvenile hormone of insects cannot be answered until the structure of the juvenile hormone is known, nor do we know at present what role these juvenile hormone substances play in groups other than insects. Nevertheless, it remains an intriguing fact that substances that act as a growth hormone for insects occur in both hydroids and cattle. It supports the view that in the course of evolution there have not been a great number of innovations at the level of small molecules since the Cambrian Era, and that the evolution of humoral mechanisms has proceeded by particular groups of animals adapting available and often ubiquitous molecules to special tasks.

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Summary

1. A new assay for the juvenile hormone of insects is described which permits detection of very small amounts of hormone activity.

2. Using this procedure extracts of a variety of invertebrates were assayed for juvenile hormone activity.

3. Juvenile hormone activity was detected in Hydrozoa, Polychaeta, Oligochaeta, Lepidoptera, Coleoptera, Decapoda, Holothuroidea, and Balanoglossida.

4. The richest source of juvenile hormone outside of insects was the eyestalk of Crustacea and it is suggested that the juvenile hormone plays a role in crustacean physiology.

5. The significance of these findings is discussed in relation to the evolution of humoral mechanisms.

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A STUDY OF SOME EFFECTS OF GAMMA RADIATION ON THE ADULTS AND EGGS OF AEDES AEGYPTI

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The studies to be reported in this paper were undertaken to evaluate some of the biological effects of ionizing radiation on the mosquito *Aedes aegypti*. This phase of the work describes in particular the effects produced by gamma radiation on the fertility and reproductive capacities of the males and females of this mosquito species, detailing as well, additional studies on the effects of radiation on the viability of eggs in various stages of development or age. It may be of interest to note that although there is an extensive literature on irradiation of insects, and in particular on irradiation of *Drosophila*, nevertheless, these studies have been concerned in the main with genetic effects rather than with the specific biological effects reported here.

Materials and Methods

The strain of *A. aegypti* used in these experiments was obtained originally from the U. S. Bureau of Entomology and Plant Quarantine Laboratory of Beltsville, Maryland, in June, 1945. It has been maintained since that time in this laboratory in wire screened cages, measuring $27 \times 24 \times 24$ inches, at a constant laboratory temperature of 80° F. and a relative humidity of 75 per cent. To maintain egg production the females have been allowed to gorge on guinea pigs once a week. The resulting eggs have been collected on strips of filter paper, and then conditioned by storing the wet strips in closed jars for a period of three days, after which they are dried and stored at room temperature for future use. The larvae from hatched eggs have been reared in glass jars containing approximately 2500 ml. of tap water, and have been fed on guinea pig pellets added in appropriate amounts each day. On this regimen, it usually requires 8 days for newly hatched larvae to reach the pupal stage. All the adults and eggs exposed to gamma radiation were derived from this colony.

The adults used for experimental purposes were kept in plastic cylinders of 3 inches height and 4 inches diameter, in groups of 40 males and/or 40 females, and were fed during the course of the experiments on 4 per cent sugar solutions. Experimental groups which require blood were fed exclusively on chicks, usually weighing about 300 grams. Adult mosquitoes were kept in the same cylinders during exposure to radiation, and it was possible to expose 6 cylinders simultaneously in the cobalt 60 irradiator used for these experiments. The strips of paper holding the eggs were placed in Petri dishes, and following exposure to radiation were stored

[1] The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

in the same dishes. To insure a maximal hatch, irradiated eggs were kept immersed in water for at least 72 hours.

The mosquitoes were exposed to gamma radiation at an approximate rate of 450 r per minute in air, in a cobalt 60 irradiator of 4 pi geometry.

Results

Irradiation of the male

In the first series of experiments designed to determine the effects of varying dosages of gamma radiation on male *A. aegypti*, 40 male and 40 female mosquitoes were allowed to emerge separately into plastic cylinders and on the fourth day following emergence, the males were exposed to gamma radiation in the cobalt irradiator. On either the first, the eighth, or the fifteenth day following exposure to radiation, the males were then placed in the cylinders containing the females, and shortly after pairing the females were allowed to gorge themselves on normal chicks. The paired mosquitoes then remained together for the duration of the experiment. Control groups were handled in exactly the same manner except that they, of course, received no radiation.

At each dosage level, two to four trials were conducted for both the experimental and control groups, and the number of eggs laid by each group and the percentage of the eggs which hatched were estimated as accurately as possible. Following hatching, the larvae from the experimental groups were grown in the usual manner to determine whether or not they would develop into viable adults. If adults finally developed, these, in turn, were given a blood meal and allowed to mate and the resulting eggs were immersed in water as a final test of parental fertility. Finally, many of the male mosquitoes were dissected at suitable intervals following exposure to radiation in order to determine the presence and condition of the spermatozoa in the seminal vesicles. At the same time, non-irradiated females which had been paired with irradiated males were also dissected to determine the presence and condition of spermatozoa within the spermathecae.

Table I summarizes most of the data concerning the effects of gamma radiation on male *A. aegypti*. In dosages ranging from 1000 r to 30,000 r, exposure to radiation produced no significant effect on the number of eggs laid in those groups in which the males were irradiated one day before they were mated to normal females. Both the control, or non-irradiated animals, and the experimental ones deposited about the same total number of eggs. In the groups in which the males were irradiated but were not paired and offered a blood meal until 8 days after radiation, however, there was evidence of some reduction in the number of eggs deposited among those in which the males had received 30,000 r, while among the groups paired and given a blood meal 15 days after the males had been irradiated, it required only 20,000 r to produce a significant reduction in the number of eggs deposited by the females. In these groups the pattern of egg-laying activity resembled that of 5-day-old and 19-day-old virgin females, allowed to take a blood meal. Such non-inseminated females produced about 20 per cent less the number of eggs than that produced by inseminated females of comparable age, and in addition, the oviposition period of such females extended over significantly longer periods of time.

TABLE I

The oviposition pattern of normal female Aedes aegypti mated to males exposed to varying doses of gamma radiation

Dosage (r)	Days post-irradiation, mating and blood-meal								
	1			8			15		
	No. eggs laid	Length oviposition period (days)	Per cent hatch	No. eggs laid	Length oviposition period (days)	Per cent hatch	No. eggs laid	Length oviposition period (days)	Per cent hatch
0	1060	11	100	1250	13	100	1100	16	100
1,000	1440	12	84	900	10	95	950	9	98
2,500	940	10	64	1150	12	85	1400	13	46
3,500	880	14	43	1000	13	46	1200	17	38
5,000	1150	12	5	1050	13	8	1600	26	9
7,500	1110	9	5*	1230	11	6*	1400	21	2*
10,000	1530	14	1	1000	14	2	1150	25	2
20,000	900	14	0	1100	22	0	800	41	0
30,000	1000	11	0	850	40	0	750	57	0

* Fertile F-1 progeny reared.

The length of the oviposition period was markedly affected by both the quantity of radiation applied to the males and the interval between exposure to radiation and mating. In 27 control groups given a blood meal and allowed to mate on the fifth day following emergence, eggs were deposited for periods ranging from 8 to 15 days, averaging 11 days, after the blood meal. In 27 corresponding experimental groups, in which males exposed to doses of radiation ranging from 1000 r to 30,000 r on the fourth day following emergence were mated on the next day and the females then given a blood meal, the length of the oviposition period differed very little from that of the control animals. In a second series, in which the females of 25 control groups were given a blood meal and allowed to mate on the twelfth post-emergence day, eggs were deposited for periods ranging from 8 to 16 days, averaging 13 days, after the blood meal. In the corresponding experimental groups, in which the males were irradiated on the fourth post-emergence day and mated 8 days later, there was no significant difference in the length of the oviposition period among those groups in which the males received radiation dosages ranging up to 10,000 r. At dosages of 20,000 r, however, eggs were deposited up until 22 days following the blood meal, and in the groups in which the males had received 30,000 r, the females continued to deposit eggs for periods as long as 40 days after the blood meal.

Increases in length of the oviposition period were even more apparent in the third set of experimental groups, in which the males and females were paired and the females then given a blood meal 14 days after the males had been exposed to radiation. In this series, 24 control groups in which non-irradiated mosquitoes were mated and given a blood meal on the nineteenth day after emergence, eggs were deposited for periods ranging from 9 to 25 days, averaging 16 days, after the

blood meal. In the experimental groups in which the males had received radiation dosages up to 3500 r on the fourth day and mated 15 days later with normal females, the length of the oviposition period was similar to that of the control groups cited above. At dosages of 5000 r to 10,000 r, however, eggs were deposited for periods which averaged 24 days after the blood meal, while at 20,000 r dosages, this period was extended to an average of 41 days, until at 30,000 r dosages the females continued to lay eggs for periods averaging 57 days. It is of interest to note that along with the increase in the egg-laying period, there was a corresponding increase in the number of days in which the females laid eggs. Thus, in the series in which males exposed to 30,000 r were mated to normal females 8 days later, and in which the oviposition period was extended to 40 days, eggs were actually laid during this interval on 27 different days as compared to 9 days on which normal females mated to normal males laid eggs. In the most extreme case, in which males exposed to 30,000 r were mated 15 days later, the females laid eggs on 33 different days as compared to 10 days for the control females in that series.

In view of these results, it was necessary to determine, first, the effects of gamma radiation on the spermatozoa in the male mosquito. For this purpose, males were dissected and the seminal vesicles examined at appropriate intervals following exposure to varying dosages of radiation. At dosages up to 10,000 r, there was no evident effect on motility, nor any evidence of morphological damage to the spermatozoa for at least 40 days following exposure to radiation. Similarly, at dosages of 20,000 r there was no evidence of loss of motility or morphological damage to the spermatozoa for a period of 25 days. After 25 days, however, during which period the mosquitoes began dying from the effects of the radiation, the spermatozoa were found to be in various stages of fragmentation and degeneration. In males exposed to 30,000 r, loss of motility and deterioration of the spermatozoa did not occur until 20 days after irradiation, at which time again the mosquitoes began dying from the effects of the irradiation. With the radiation dosage increased to 50,000 r, spermatozoa remained motile and normal in appearance for only about 10 days, at which time deterioration of the spermatozoa and death of the adults began to occur simultaneously as usual. In general, therefore, spermatozoa remained normal in appearance and motile for about as long as the mosquitoes themselves were able to survive the various doses of radiation.

Secondly, to determine whether insemination had occurred, and to observe the condition of the spermatozoa in inseminated females, the spermathecae of normal females mated to irradiated males were examined at appropriate intervals after the animals had been paired. As a result, it was found that all the females paired with males one day after the males had been exposed to 20,000 r contained motile spermatozoa normal in appearance for at least two weeks after mating. On the other hand, only about one-half the females mated 15 days after the males had been exposed to 20,000 r contained normal spermatozoa the third day after mating. In the remainder, either the spermatozoa were in fragments or, more usually, there were no spermatozoa present. And again, the spermathecae of all the females mated a day after the males had been exposed to 30,000 r contained normal appearing spermatozoa for at least two weeks after pairing. If, however, pairing was delayed until 15 days after exposure of the males to radiation, none of the spermathecae of the 25 specimens were found to contain spermatozoa when examined the day after mating. Thus,

these data suggest that the reduction in egg production and increase in the period of oviposition evidenced when mating was delayed following exposure of the males to radiation was due simply to inability of more and more of the deteriorating males to copulate successfully rather than to any lack of motile spermatozoa.

That morphological integrity and motility of the spermatozoa, or significant changes in the egg-laying pattern of females mated to irradiated male mosquitoes, are inadequate criteria for determining the biological effects of gamma radiation on the fertility of the males, is shown by observations on the hatching of eggs laid by normal females mated to males exposed to varying doses of radiation, and the viability of the larvae emerging from such eggs. Thus, as shown in Table I, although in all the groups mated at various intervals following irradiation of the males, the females continued to lay eggs in considerable numbers, nevertheless, the number of larvae hatching from these eggs become progressively less with increasing exposure to irradiation of the males.

In view of the fact that even under well controlled conditions of temperature and humidity, there was considerable variation in the percentage of eggs hatching from eggs produced from normal matings, it was necessary for purposes of comparison, to consider the hatch from a given number of normal, control eggs as a 100 per cent hatch. And again because of variations in the hatch from a given collection of eggs, differences in the degree of hatch between eggs from control and experimental were not too apparent until the disparity in hatching between the two groups became quite considerable.

As shown in Table I, in all three groups mated at various intervals following irradiation of the males, the first real evidence of any appreciable reduction in the percentage of larvae hatching from a given number of eggs was in those groups in which the males were exposed to at least 2500 r. In the groups in which the males had received 3500 r there was little further reduction in hatch except in the group in which the males were not mated with normal females until 15 days after their exposure to radiation, in which case the hatch was only 18.4 per cent of that in the control group. There was, however, a sharp decrease in the hatch from eggs derived from groups in which the males had received 5000 r, so that in the ones mated 1 and 8 days following exposure to radiation, only 5.3 per cent and 7.6 per cent, respectively, of the eggs hatched, as compared to the controls. On the other hand, as if to illustrate again the variations in hatching which may occur, in the third remaining group which had been mated 15 days following exposure to radiation, 20.0 per cent of the eggs produced hatched into viable larvae.

There was little further reduction in the proportion of eggs hatching among the groups in which the males had received 7500 r. In the groups in which the males had been exposed to 10,000 r, however, only slightly over 1 per cent of the eggs hatched, while in the groups in which the males had received 20,000 r and 30,000 r, no larvae ever hatched from the eggs which were produced.

The fact that the number of viable eggs produced was the same, at any given dosage, in the groups mated 15 days after irradiation as in the groups mated the day following exposure, indicates that once a sperm was damaged to the extent that it was no longer capable of fertilizing an egg, there was no further recovery and the injury remained permanent, whereas if a sperm escaped such lethal injury initially there was no further physiological deterioration and it remained uninjured and

capable of fertilizing an egg. Since there was no decrease or increase in the production of viable eggs to indicate either deterioration or recovery, neither of which process could be expected to proceed at the same rate, it appears reasonable to assume that gamma irradiation has a kind of all-or-none effect on the spermatozoa of *A. aegypti,* and that the extent of this effect will depend upon the level of radiation that has been administered. Further, the data cited above lend further support to the assumption that spermatogenesis is not a continuing process during the adult life of the male *A. aegypti.*

It is of interest to note that the larvae hatching from the matings described above could be reared successfully and the resulting adults, when mated themselves, produced fertile, viable eggs, providing there were sufficient larvae present to eliminate the cultivation problems that arise when larval colonies are too small. Thus, from matings in which the males had received dosages up to 3500 r, larvae were reared with comparative ease, but at dosages of 5000 r and 7500 r, when only very few larvae were available, rearing them to adulthood became a major problem of cultivation. Even at these dosages, however, the few larvae that were finally grown mated successfully as adults and produced viable progeny. Although several attempts to rear the isolated larvae hatching from matings in which the males had received 10,000 r were unsuccessful, there is some reason to believe that with enough care such larvae could be grown to adults, and in such a case the adults would in all likelihood produce viable progeny.

Irradiation of the females

In these experiments, designed to measure the effects of varying doses of gamma radiation on the oviposition habits of female *A. aegypti,* again 40 male and 40 female mosquitoes were allowed to emerge separately into plastic cylinders except that in this case on the fourth day following emergence, the females were irradiated and then paired with normal male mosquitoes on the first, eighth, or fifteenth day following exposure to radiation and offered a blood meal soon after they had been mated. The resulting eggs were collected, counted, then incubated as usual in a saturated atmosphere for five days, and finally hatched. The larvae were grown to adults and mated, and the resulting eggs were then collected and allowed to hatch as evidence of fertility of the F-1 adults. It may be noted here that as in the case of eggs produced from matings in which the males were irradiated, if the eggs hatched they could usually be grown to adults.

As shown in Table II, there was no significant reduction in the number of eggs produced by females exposed to 1000 r, or 2000 r, and mated the following day. There was, however, a significant reduction in the number of eggs produced by females exposed to 2500 r, while the females exposed to doses as high as 10,000 r produced only a few isolated eggs. On the other hand, in the groups mated 8 and 15 days following irradiation, although again there was little or no reduction in the number of eggs produced by females exposed to doses up to 2000 r, egg production dropped off sharply in females exposed to 2500 r, while females exposed to 5000 r laid no eggs at all. In general, therefore, the longer the mating of females exposed to radiation doses above 2000 r was delayed, the fewer the eggs they produced, and the lower the dosage required to eliminate egg production entirely. Similarly, the fewer the eggs that were produced, the less the proportion of them that eventually

hatched into viable larvae. Thus, whereas 64.9 per cent of the eggs laid by females exposed to 3000 r and mated one day later hatched, only 25.6 per cent of the eggs laid by females exposed to the same dosage but mated 15 days later hatched, and similarly while 67.0 per cent of the eggs laid by females exposed to 3500 r hatched when they were mated 24 hours later, only 24.0 per cent of the eggs hatched when mating was delayed 15 days. And, finally, although a small percentage of the eggs laid by females exposed to 5000 r and mated one day later hatched into viable larvae, none of the females exposed to 5000 r or higher laid any eggs at all when mated 8 or 15 days following exposure.

These data indicate that in female *A. aegypti* exposed to radiation above certain threshold levels, in this case approximately 2000 r, the ovaries are not only incapable of recovering from the injury produced by radiation, but rather that the functional activity of the ovary becomes progressively further impaired during the interval following exposure. It may be noted in passing that unlike the case of normal females mated to males exposed to radiation, in which the reduction in egg production resulted in lengthening of the period of oviposition, in this case the reduced egg production of irradiated females mated to normal males resulted instead in significant lessening of the oviposition period, indicating again the loss in functional activity of the impaired ovaries.

To determine whether irradiated females could mate successfully and whether spermatozoa could survive in them, groups of virgin females were exposed to doses of 20,000 r and 30,000 r, paired with normal males either 1 or 15 days later, and then dissected at suitable intervals following mating. The spermathecae of all the females exposed to 20,000 r, and paired the day following radiation, contained motile spermatozoa for a period of two weeks after mating had occurred. However, when

TABLE II

The oviposition pattern of female Aedes aegypti exposed to varying doses of gamma radiation and then mated, at different intervals following irradiation, to normal males

Dosage (r)	Days post-irradiation, mating and blood meal					
	1		8		15	
	No. eggs laid	Per cent hatch	No. eggs laid	Per cent hatch	No. eggs laid	Per cent hatch
0	1130	100	1070	100	1100	100
1,000	1050	100	1100	100	1000	100
1,500	1350	71	1050	90	1000	91
2,000	1000	87	1000	90	700	78
2,500	700	87	830	62	650	32
3,000	650	65	800	42	400	26
3,500	550	43	600	35*	190	24*
5,000	25	13*	0		0	
7,500	10	0				
10,000	10	0				
20,000	0					

* Fertile F-1 progeny reared.

mating was delayed until 15 days after irradiation, an average of only 8 out of 10 females were found to contain spermatozoa during the ensuing two weeks. Similarly, the spermathecae of all females exposed to 30,000 r contained spermatozoa for at least 2 weeks if pairing took place the day after irradiation, but if mating was delayed until 15 days after exposure to radiation, then only an average of 4 out of 10 females dissected at intervals during the subsequent 2 weeks contained spermatozoa. From these results, it would appear as if females exposed to high doses of radiation are capable of mating shortly after exposure and that spermatozoa will apparently survive in them for at least two weeks, but if mating is delayed too long, radiation injury to the mosquito as a whole progressively reduces the chances of successful mating

The second series of experiments was designed to ascertain whether viability or production of eggs was influenced by insemination of the females prior to radiation rather than subsequent to radiation as in the previous experiments. Accordingly, to assure insemination 40 females and 40 males were allowed to emerge into the same cylinders and were maintained together for four days following emergence. At the end of this period the males were removed and the females were exposed to varying doses of radiation. One, eight and fifteen days after irradiation these females were allowed to take a blood meal and the resulting eggs were collected, incubated and hatched. The data presented in Table III indicate that females inseminated prior to irradiation and given blood at various subsequent intervals laid about the same number of eggs as females inseminated at similar intervals following exposure to radiation, as shown previously in Table II. Similarly, as in the previous experiment, the number of eggs produced decreased significantly at

TABLE III

The oviposition pattern of female Aedes aegypti mated first to normal males, exposed to varying doses of gamma radiation after mating, and then allowed to take a blood meal at different intervals following irradiation

Dosage (r)	Days post-irradiation, blood meal					
	1		8		15	
	No. eggs laid	Per cent hatch	No. eggs laid	Per cent hatch	No. eggs laid	Per cent hatch
0	1300	100	1280	100	1000	100
1,000	1400	75	1300	68	1250	86
1,500	1450	55	1220	68	1320	60
2,000	1350	67	1150	61	900	57
2,500	1180	67	910	33	630	44
3,000	600	34	620	15*	670	14
3,500	380	3	200	0	520	7*
5,000	30	2*	0		0	
7,500	30	0	0		0	
10,000	40	0				
20,000	0					

* Fertile F-1 progeny reared.

2500 r to 3000 r, and except for those females which received a blood meal one day after irradiation, egg-laying was almost entirely inhibited at doses 5000 r or above.

On the other hand, the number of eggs which hatched from among those laid by females inseminated prior to exposure to radiation was consistently below the number which hatched from among those laid by females inseminated subsequent to exposure to radiation. Thus, in the groups in which the females were mated and then exposed to as little as 1000 r there was a significant reduction in the number of eggs hatching from among those produced, while there was no reduction in the number of eggs that hatched of those produced by females inseminated following exposure to the same dosage, whereas 43 per cent of the eggs laid by females inseminated subsequent to exposure to 3500 r hatched out, only 3 per cent of the eggs produced by females inseminated prior to exposure hatched into viable larvae. Obviously, insemination of females prior to exposure to radiation reduces the viability of the eggs simply by introducing an additional source of injury, namely, injury to the spermatozoa in addition to the effect on the ovaries.

As before, it was possible to rear fertile F-1 progeny whenever enough larvae hatched out from the eggs. Thus, fertile F-1 progeny were reared from females inseminated either before or after exposure to doses as high as 5000 r and then given a blood meal the day after radiation. At dosages above 5000 r although a few eggs were produced, as shown in Tables II and III, none hatched. On the other hand, no eggs were produced by females exposed to 5000 r but not allowed a blood meal until 8 and 15 days following exposure to radiation, and fertile progeny were reared only from females receiving 3500 r.

In female *A. aegypti* ovarian activity is apparently suspended until the animal takes a blood meal. Following a blood meal development of the egg proceeds, fertilization takes place providing the spermathecae contain spermatozoa, and finally oviposition begins. At the temperatures maintained in this laboratory, female *A. aegypti* will begin to produce fertile eggs approximately 48 hours after a blood meal has been taken.

The next series of experiments was designed to study the effects of radiation on the cycle of events occurring in fertilized females between the time blood is ingested and oviposition begins. Thus, in these experiments again 40 males and 40 females were allowed to remain together for 4 days following emergence, then at the conclusion of this period, the males were removed and the females were given a blood meal. Subsequently, at intervals of 4, 24 and 42 hours after the blood meal the females were exposed to varying doses of radiation.

The results shown in Table IV indicate that the various doses of radiation had their greatest effect on egg production in those groups irradiated 4 hours after a blood meal, a significantly lesser effect in the groups irradiated 20 hours later, and the least effect in the groups irradiated 42 hours after the blood meal. Thus, whereas the group exposed to radiation 4 hours after it had received a blood meal laid a total of 2305 eggs and no eggs were produced by any females exposed to more than 10,000 r, the group exposed 24 hours after it had engorged laid 12,610 eggs and a few eggs were produced by females receiving as much as 70,000 r. Finally, in the group irradiated 42 hours after the blood meal, 11,010 eggs were laid and it required exposures in excess of 100,000 r to inhibit egg production com-

TABLE IV

Oviposition and egg viability of inseminated female Aedes aegypti exposed to gamma radiation at various intervals following a blood meal

Dosage (r)	Hours post-blood-meal, irradiation					
	4		24		42	
	No. eggs laid	Per cent hatch	No. eggs laid	Per cent hatch	No. eggs laid	Per cent hatch
0	1200	100	1400	100	1480	100
2,500	1060	69	1380	45	1270	29
3,000	600	53	1400	38	1400	13
3,500	230	31	1660	30	1340	7*
5,000	160	7*	1590	10*	1280	1
7,500	110	8	1720	6	1480	0
10,000	40	0	1970	0	1180	0
20,000	0		970	0	980	0
70,000			10	0	310	0
80,000			0		130	0
100,000					10	0
110,000					0	

* Fertile F-1 progeny reared.

pletely. It would appear from these data that the early phases of the complicated sequence of physiological events leading to egg production are highly sensitive to radiation injury but that once the mechanism has been established and has proceeded to some specific developmental stage, oviposition will take place in spite of excessive radiation injury.

On the other hand, the data show that although the mechanisms responsible for egg production become more resistant as development proceeds, the eggs themselves become more sensitive to the effects of radiation as they mature during the preoviposition period. Thus, in the groups irradiated 4 and 24 hours following the blood meal, about 8 and 6 per cent, respectively, of the eggs produced by the females exposed to 7500 r hatched into viable larvae but in the groups irradiated 42 hours after the blood meal, only about 1 per cent of the eggs produced by females exposed to 5000 r hatched, while none of the eggs produced by females exposed to 7500 r proved to be viable. And similarly, in the lower dosages, the hatch from eggs produced by females exposed to radiation 4 hours after the blood meal was far more abundant than the hatch from eggs produced by females exposed 42 hours later.

Viable, fertile F-1 progeny were obtained from the eggs produced by females exposed to 5000 r 4 and 24 hours after the blood meal, but the few larvae from eggs of females exposed to 6000 r and 7500 r died shortly before the pupal stage. In the groups irradiated 42 hours following the blood meal, viable, fertile F-1 progeny were reared from the larvae that hatched from eggs deposited by females exposed to 3500 r, but again the few larvae hatching from eggs deposited by females exposed to 5000 r died during cultivation. There is reason to believe that with greater numbers of larvae available, it might have been possible to rear adults from

the groups in this experiment in which the larvae died during cultivation. Nevertheless, as before, whenever larvae from irradiated parents could be grown to adulthood, the progeny always proved to be fertile.

Irradiation of eggs

In the first experiments designed to determine the effects of ionizing radiation on the eggs of *A. aegypti*, eggs of two different age groups were selected for study. The first group consisted of eggs that were 25 to 50 hours old in which embryonation had not been completed, while the second group consisted of eggs approximately 400 hours old in which such development had long been completed so that the eggs would normally hatch at once upon immersion in water. In this laboratory, properly conditioned, normal eggs usually require 65 hours from the time they are laid until they hatch. Since the dose rate was about 450 r per minute, the eggs in the first groups were in the cobalt irradiator for periods not above 100 minutes, but the eggs of the second group, which required extraordinarily high doses of radiation to inhibit hatching, had to be kept in the cobalt irradiator for periods up to about 23 hours. Following exposure to radiation, the eggs were kept at insectary temperatures for 1, 8, or 15 days before being allowed to hatch. About 2000 eggs were used for each exposure and there were from 4 to 8 different exposures for each age category. To assure hatching, if hatching was to occur at all, eggs were kept immersed for 72 hours whenever necessary.

The data presented in Table V indicate that the hatching capabilities of the eggs which were 25 to 50 hours old when irradiated, and which have been designated as 2 days old, were far more susceptible to radiation injury than the eggs which were 16 days old when they were irradiated. Thus, whereas in the former it required only 10,000 r to reduce the hatch by 50 per cent, in the latter it required dosages ranging from 20,000 r to 100,000 r to reduce hatching to the same extent. And again, while a radiation dose of 20,000 r sufficed to inhibit completely

TABLE V

The effect of gamma radiation on the hatch of 2-day-old and 16-day-old Aedes aegypti embryos

Dosage (r)	2-day-old eggs (25–50 hrs.) Days post-irradiation, per cent hatch			16-day-old eggs Days post-irradiation, per cent hatch		
	1	8	15	1	8	15
2,500	100	100	100	100	100	100
5,000	100	100	80	100	100	80
7,500	75	75	75	100	80	80
10,000	50	50	50	80	75	75
15,000	25	25	25	80	75	75
20,000	0	0	0	80	75	50
30,000				75	50	25
100,000				50	20	10
150,000				25	0	0
200,000				10		
500,000				10		
550,000				0		

the hatching of 2-day-old eggs, which were being exposed while embryonic development was still in progress, it required doses ranging from 150,000 r to as much as 550,000 to eliminate entirely the hatching of 16-day-old eggs in which, of course, embryonic development had been completed prior to exposure. In addition, as shown in Table V, it was found that storage of 2-day-old eggs following exposure to radiation produced no further deterioration in the ability of the eggs to hatch but that storage of 16-day-old irradiated eggs resulted in marked deterioration, at the various dosage levels, in the ability of the eggs to hatch successfully. Thus, in 2-day-old eggs, as many eggs hatched out when immersed 15 days subsequent to doses of 10,000 r and 20,000 r as had hatched following immersion only one day following exposure, but with 16-day-old eggs, none of the eggs exposed to 150,000 r hatched out at the end of 15 days although 25 per cent of them had hatched when immersed the day following exposure. And again, although 10 per cent of the eggs which had received 500,000 r hatched when immersed the day following exposure, a comparable hatch was obtained from eggs which had received only 100,000 r when immersion was delayed 15 days following exposure to radiation.

It is of interest to note that although it required enormous doses of radiation to destroy the ability of the larvae to hatch, and there were wide differences between 2-day-old and 16-day-old eggs in the amount of radiation required to produce this effect, nevertheless, fertile F-1 adults could not be produced from either 2-day-old or 16-day-old eggs exposed to more than 2000 r, whether hatched 1, 8, or 15 days following exposure. Thus, only about 50 per cent of the larvae from either 2-day-old or 16-day-old eggs exposed to 1000 r developed into adults, almost all of which, however, were able to mate successfully. From eggs exposed to 1500 r, no more than about 10 per cent of the larvae developed into adults, and of these probably one-half were able to mate. From eggs exposed to 2000 r only about one per cent of the larvae developed into adults, and of these approximately one-third were able to mate and produce viable eggs. The remaining adults in these groups were usually too feeble even to feed and most of them died almost immediately after emergence. However, all the males examined in such cases were found to contain motile spermatozoa. It may be noted, too, that a considerable proportion of the mortality in these groups occurred after ecdysis when the animals, apparently too weak to fly off properly, simply fell back into the water. The larvae developing from eggs exposed to 2500 r died during either the fourth larval instar or the pupal stage, while very few larvae from eggs exposed to 3000 r survived beyond the third instar, but of those that did, all died during the fourth larval stage.

Finally, experiments were designed to determine the effects of varying doses of radiation on the hatching ability of eggs of *A. aegypti* of various ages. For this purpose the eggs were kept at room temperature for periods ranging from less than 24 hours up to 180 days, exposed to radiation and then immersed for hatching 4 days after having been irradiated. Immersion had to be delayed 4 days in order to insure embryonation of the eggs which were irradiated while they were still less than 65 hours old. About 2000 eggs were used in each trial and every group of irradiated eggs was matched by a control, or non-radiated group of eggs from the same adult colony. The experiment was terminated with 180-day-old eggs, since it was found that the hatching of control eggs was so poor after that period

that it was almost impossible to isolate any effect of radiation. No hatch whatsoever could be obtained from 270-day-old control eggs.

It is evident from the data presented in Figure 1 that resistance or susceptibility to radiation was related to the age of the egg. During the first 24-hour period following oviposition, a time of active embryonic development, the eggs were particularly susceptible to radiation damage, so that as little as 800 r caused a 50 per cent reduction in hatch while 6000 r inhibited hatching entirely. By the time the eggs were 48 hours old and embryonic development was nearly complete, however, their resistance had increased so markedly that it required 7500 r to produce a 50 per cent reduction in hatch and 25,000 r to eliminate hatching entirely. During the period in which hatching would have normally occurred (third day), resistance was still further increased so that it required 30,000 r to effect a 50 per cent reduction in hatch. Resistance to radiation increased to maximal dosage levels in four- and five-day-old eggs so that it required 75,000 r to produce a 50 per cent reduction in hatch, and at least 130,000 r to inhibit hatching completely. Further aging gradually lessened resistance, however, until by the 180th day after oviposition, administration of 4000 r reduced the hatch by 50 per cent although it still required some 75,000 r to eliminate hatching completely. Progeny were reared from eggs of various ages irradiated at dosages ranging from 1000 r to 2500 r and there was no discernible evidence to indicate that the age of the egg influenced in any way

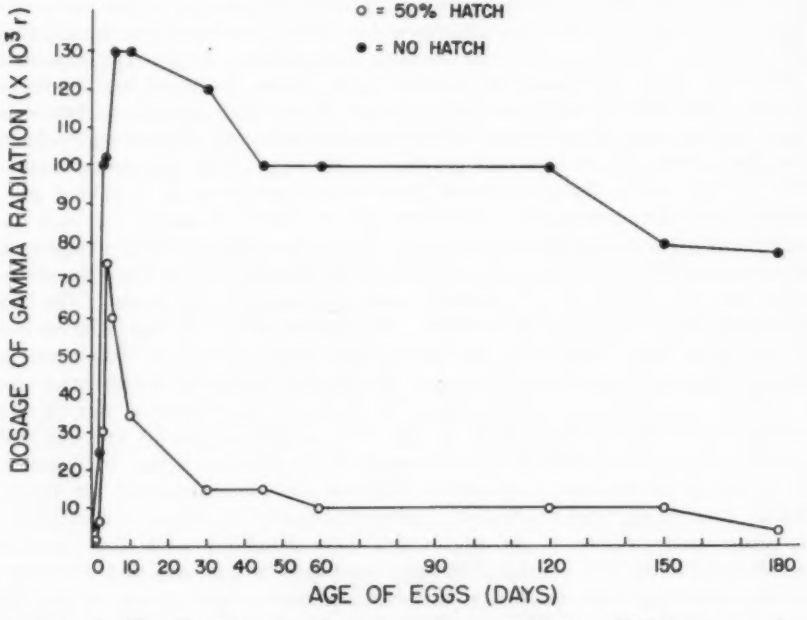

FIGURE 1. The relation between the radiation dosage required to inhibit hatching and the age of the eggs of *Aedes aegypti*.

the viability of the F-1 progeny. Attempts to rear fertile progeny from these irradiated eggs produced results almost identical to the results obtained with 2-day-old and 16-day-old eggs except that in this case, after numerous attempts, a few adults were finally obtained from eggs exposed to 2500 r, which did mate and lay fertile eggs. Thus, again, although the dosage required to impair the impetus to hatch varied with the age of the egg, the dosage required to destroy the viability of the egg was constant regardless of age. It is of interest to note, again, that the dosage was directly related to the length of time larvae developed or survived following exposure to radiation, in that the higher the dosage the quicker larval development was arrested and the larvae died.

Summary

1. It has been shown that when normal *A. aegypti* females are mated and given a blood meal 24 hours after exposure of the males to gamma radiation in doses up to 30,000 r, egg production is not significantly affected. If, however, mating is delayed 8 or 15 days following irradiation of the males, egg production decreases and the period of oviposition increases apparently because fewer males are able to copulate even though they still contain motile spermatozoa.

2. Although eggs continue to be produced in some quantity whether mating is immediate or delayed, fewer larvae hatch from eggs produced by females mated to males exposed to 2500 r, while a very few larvae hatch from eggs produced from matings in which the males received 10,000 r. However, it was possible to grow larvae successfully to fertile adults capable of mating and producing viable eggs only from matings in which the males had received a maximum of 7500 r.

3. It has been shown, too, that the egg production of female mosquitoes, exposed first to gamma radiation and mated 24 hours later to normal males, is significantly reduced among those receiving 2500 r, and almost entirely eliminated among those exposed to 10,000 r. However, larvae which could be grown to fertile adults were obtained only from eggs produced by females exposed to a maximum of 5000 r. When mating was delayed, no eggs were produced by females exposed to doses in excess of 3500 r, although viable larvae hatched from eggs of females exposed to 3500 r.

4. Females inseminated prior to being exposed to radiation produced approximately the same number of eggs at the various dosage levels as females inseminated subsequent to exposure. However, significantly fewer larvae hatched from these eggs than from the eggs laid by females inseminated subsequent to exposure.

5. To determine the effects of radiation during the cycle of egg development which occurs in *A. aegypti* following a blood meal, inseminated females were exposed to gamma radiation at various intervals following engorgement. It was found that egg production was almost entirely inhibited in females exposed to 10,000 r 4 hours after the blood meal, whereas it required in excess of 100,000 r to inhibit egg production in females in which exposure had been delayed 42 hours after the blood meal. On the other hand, although it required higher and higher doses of radiation to inhibit egg production the longer irradiation was delayed, nevertheless, the eggs became more and more sensitive to radiation as they matured within the body. Thus, whereas viable larvae resulting in fertile adults developed from eggs produced by females exposed to 5000 r 4 or 24 hours after the blood

meal, viable larvae could be obtained from the eggs of females exposed to only 3500 r when exposure was delayed 42 hours.

6. Finally, it has been shown that although the dosage required to inhibit hatching of the eggs of *A. aegypti* exposed to gamma radiation varied enormously according to the age of the egg, nevertheless, eggs exposed to doses in excess of 2000 r, regardless of age, could not be grown to adults. Again, however, as in the experiments in which either males or females were exposed to radiation, whenever larvae could be grown successfully to adults, the resulting adults proved to be fertile and capable of producing viable eggs if they were physically capable of mating.

QUATERNARY AMMONIUM BASES IN THE COELENTERATES [1]

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The stinging organelles or nematocysts of coelenterates appear to serve two functions. By injecting an irritating substance they serve as effective weapons of defense, while accompanying paralyzing action, probably by a different agent or combination of agents, is useful in quieting prey in the process of feeding. In spite of a considerable amount of work over the past fifty years, the chemical nature of the nematocyst toxin is still unknown. Some of the earlier literature has been summarized elsewhere along with an account of some recent work (Welsh, 1956).

Aqueous extracts of nematocyst-bearing tentacles of representatives of each of the three classes of coelenterates, when injected in crabs, produce a preliminary excitation. Spontaneous autotomy of legs may accompany the excitation. After a time the crabs become paralyzed and, if the dose is sufficient, they fail to recover. An injection of tetramethylammonium chloride mimics the paralyzing action of extracts, while an earlier or simultaneous injection of a salt of tetraethylammonium antagonizes the paralyzing actions both of extracts and a tetramethylammonium halide (Welsh, 1956). Since Ackermann, Holtz and Reinwein (1923) had isolated tetramethylammonium hydroxide ("tetramine") from sea anemones, the possibility existed that this substance, or some derivative, was the active paralyzing principle.

Largely through the efforts of Ackermann and co-workers several other quaternary ammonium bases have been isolated from sea anemones and chemically identified. Ackermann, Holtz and Reinwein (1924a) isolated and identified N-methylpyridinium hydroxide from *Actinia equina*, along with a compound tentatively named "actinin." Later, the same authors (1924b) presented evidence that led them to suggest that actinin was probably the alkaloid stachydrine. Ackermann (1927), however, determined actinin to be γ-butyrobetaine, and not stachydrine. Recently Ackermann (1953) found homarine and trigonelline in extracts of the sea anemone, *Anemonia sulcata*, along with an unidentified base which he first named "anemonin," but later (1954) changed to "zoo-anemonin." Evidence for the occurrence of trigonelline in the siphonophore, *Velella spirans*, had been presented earlier by Haurowitz and Waelsch (1926). Zoo-anemonin was identified as the dimethylbetaine of imidazole acetic acid by Ackermann and Janka (1953), but the correctness of the structural formula that they gave will be discussed later.

The present study began as an attempt to determine whether or not tetramine was generally present in coelenterates. Since paper chromatography was used, followed by reagents that help in the visualization of quaternary ammonium bases, it soon became obvious that several such compounds were present. The work was

[1] This investigation was supported in part by research grant B-623 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service.

extended in an attempt to identify these. Unfortunately certain of the papers of Ackermann and others were not known to us until we had spent considerable time in the identification of those bases. We have now identified, with reasonable certainty, tetramethylammonium (I), homarine (III), trigonelline (IV) and γ-butyrobetaine (V) in representatives of all three classes of coelenterates (see Fig. 1). We find what probably corresponds with Ackermann's zoo-anemonin (VI) in a horny coral and in two species of sea anemone. With the method used, we have been unable to identify N-methylpyridinium (II) in any coelenterate, although using the same method we can demonstrate its presence in certain molluscan tissues. Some spots which react as quaternary ammonium bases have not been identified.

Preliminary tests of the toxicity of the identified bases have been made.

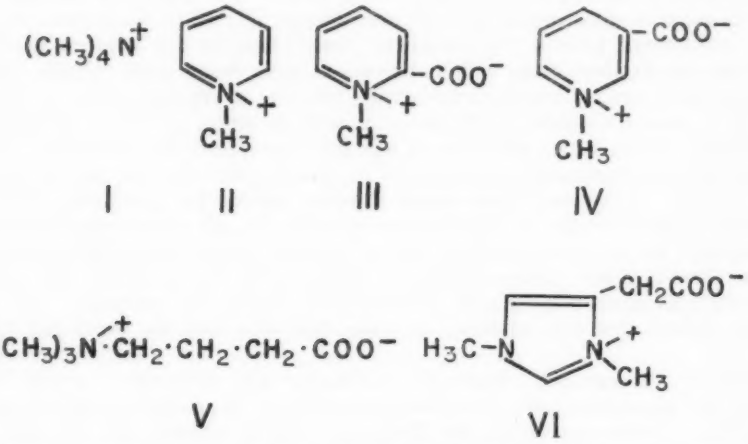

FIGURE 1. Structural formulae of compounds included in this study. I = tetramethylammonium; II = N-methylpyridinium; III = homarine; IV = trigonelline; V = γ-butyrobetaine; VI = zoo-anemonin.

MATERIALS AND METHODS

Extracts were made from the following:

Class Hydrozoa

Hydra littoralis—some supplied by Dr. W. F. Loomis; some mass cultured according to Loomis and Lenhoff (1956); others collected locally.

Physalia physalis L.—the Portuguese man-of-war, fishing filaments only; collected in Bermuda and Bimini, B. W. I.

Class Scyphozoa

Cyanea capillata (L.)—brown or red jellyfish, tentacles only; collected in Puget Sound, Washington.

Class Anthozoa

Plexaura flexuosa—a horny coral, whole animal; collected in Bermuda.
Metridium dianthus (Ellis)—sea anemone, whole animal or tentacles; collected at Nahant and Rockport, Massachusetts.
Condylactis gigantea (Weiland)—pink-tipped sea anemone, tentacles only; collected in Bermuda.

Whole animals were macerated in a Waring Blendor and 4–5 volumes of acetone added. Tentacles were cut off and placed in 4–5 volumes of acetone. The tissues in acetone were stored in a refrigerator. When needed, a given volume (20 or 25 ml.) of the acetone extract was decanted, filtered and the acetone removed under reduced pressure. The remaining material was dried and washed with about 10 ml. of petroleum ether, the bases were then taken up in 1 or 2 ml. of 95% ethanol for chromatography. No attempt was made to secure quantitative yields but the results give a good idea of the relative amounts of different bases that were extracted from a given species.

Extracts and knowns were chromatographed using wide strips of Whatman No. 3 MM filter paper. After trying a variety of acidic and basic solvent systems we found that the most satisfactory separation was obtained with a mixture of 95 parts of 95% ethanol and 5 parts of ammonium hydroxide (28%), as recommended by Bregoff, Roberts and Delwiche (1953). When two-dimensional chromatograms were run, the second solvent system was n-butanol-acetic acid-water (10:3:8–9). The jars were allowed to saturate for at least seven hours and the papers equilibrated 2–3 hours. The ascending method was used. Jars were kept in a chamber in which the temperature was maintained at 25° C. ± 1°. The most satisfactory chromatograms were obtained after runs of 9–10 hours. After drying, the chromatograms were examined under ultraviolet light (short-wave "Mineralight") and any ultraviolet absorbing areas outlined with pencil. Of the several reagents used to visualize the areas occupied by quaternary ammonium compounds, the most generally satisfactory was Dragendorff's solution ($KBiI_4$ reagent) as modified and used by Bregoff, Roberts and Delwiche (1953).

To identify zoo-anemonin, a solvent system consisting of n-butanol-dioxane-water in the proportions of 4:1:5, was also used to permit comparison of the Rf value with that obtained by Ackermann and Janka (1953).

In order to make more certain the identification of homarine and trigonelline, both of which absorb strongly in the ultraviolet, absorption spectra of eluates were compared with those of synthetic compounds using the Cary recording spectrophotometer. Rather large amounts of extracts were placed on paper and run with ethanol-ammonia solvent. Ultraviolet absorbing areas were outlined and a strip was cut from one side for development with $KBiI_4$. The desired areas were cut out and eluted with distilled water. They were appropriately diluted and absorption spectra were obtained. We are greatly indebted to Mr. and Mrs. Paul Brown for their cooperation in this part of the study.

The toxicities of tetramethylammonium bromide, N-methylpyridinium, homarine, trigonelline, γ-butyrobetaine and N,N'-dimethylimidazole acetic acid were determined on the fiddler crab, *Uca pugilator*, from Florida. Each was tested on one or more lots of 5 crabs, by injecting 0.02 or 0.05 ml. of a 1% solution at the base of one of the walking legs.

The known quaternary ammonium standards used in this study were from the following sources: tetramethylammonium bromide, Eastman Organic Chemicals; N-methylpyridinium bromide, kindness of Dr. J. A. Aeschlimann, Hoffmann-La Roche Inc.; homarine, kindness of Dr. E. L. Gasteiger; trigonelline, General Biochemicals Inc.; while γ-butyrobetaine was prepared from γ-carbomethoxypropyltrimethylammonium bromide (generously supplied by Dr. R. W. Fleming, Parke Davis and Co.) after the method suggested by Bregoff, Roberts and Delwiche (1953). A sample of the dimethylbetaine of imidazole acetic acid, as the hydrochloride ($C_7H_{10}O_2N_2 \cdot HCL \cdot H_2O$), was kindly furnished by Dr. D. Ackermann. A second sample was made from imidazole acetic acid (supplied by Dr. H. Bauer, National Institutes of Health) in the laboratory of Dr. R. B. Woodward. The two samples had similar melting points and similar Rf values. Dr. Woodward informs us that the structural formula for anemonin (zoo-anemonin) as given by Ackermann and Janka (1953) is in error and that the correct formula is as given in the series of structural formulae. The more descriptive name for this substance would, therefore, be N,N′-dimethylbetaine of imidazole acetic acid.

Results

Chromatograms

Extracts of tentacles of whole animals of the six selected species, representing each of the three classes of coelenterates, were chromatographed according to the procedure outlined in the section on Methods. Each extract was run many times along with one or more samples of known quaternary ammonium bases. The relative Rf values of these bases are given at the left of Figure 2. All results are for the ethanol-ammonia solvent system. It may be seen that tetramethylammonium bromide (I) gave an Rf value of 0.75; N-methylpyridinium bromide (II) an Rf of 0.64; homarine HCl (III) an Rf of 0.54; trigonelline (IV) an Rf of 0.32; and γ-butyrobetaine bromide (V) an Rf of 0.27. For each species, the compounds found and identified with reasonable certainty, with the exception of N,N′-dimethylbetaine of imidazole acetic acid (VI), are represented by shaded areas.

Tetramine was present in each of the species examined, being the only base found in *Hydra*. The two sea anemones yielded smaller amounts than the other species and in *Metridium* this spot was most distinct when an extract of tentacles, rather than of whole animal, was used. Extracts of the gorgonian, *Plexaura flexuosa*, contained relatively large amounts of tetramine, as suggested by the larger shaded area. It is of interest to note that separate extracts were made of purple and brown varieties of colonies of *Plexaura*. The chromatograms of these extracts were so similar that they are represented by the one set of spots of the four bases that were identified.

In none of the species examined did we find an indication of the presence of N-methylpyridinium. Since the methods employed have enabled us to identify this substance in extracts of certain molluscan tissues, we believe it to be absent, or present in very small amounts, in the coelenterates investigated. Homarine is a compound now known to be widely distributed among marine invertebrates (Gasteiger, Gergen and Haake, 1955). We found it in all five marine species of coelenterates examined. Although present in relatively large amount in our extracts of

Metridium, it was determined with least certainty in the pink-tipped sea anemone, *Condylactis*.

In the ethanol-ammonia solvent, the Rf values of trigonelline and γ-butyrobetaine were so similar that the spots overlapped. In the case of *Plexaura* extracts, where a relatively large amount of trigonelline was present, two-dimensional chromatograms were run. This permitted a clear-cut separation of trigonelline and γ-butyrobetaine. Trigonelline was not found in our extracts of *Physalia*, although it was identified with reasonable certainty in the other four species. Extracts of the pink-tipped sea anemone, *Condylactis*, contained large amounts of γ-butyrobetaine, while extracts of *Physalia* and *Metridium* appeared to lack this substance.

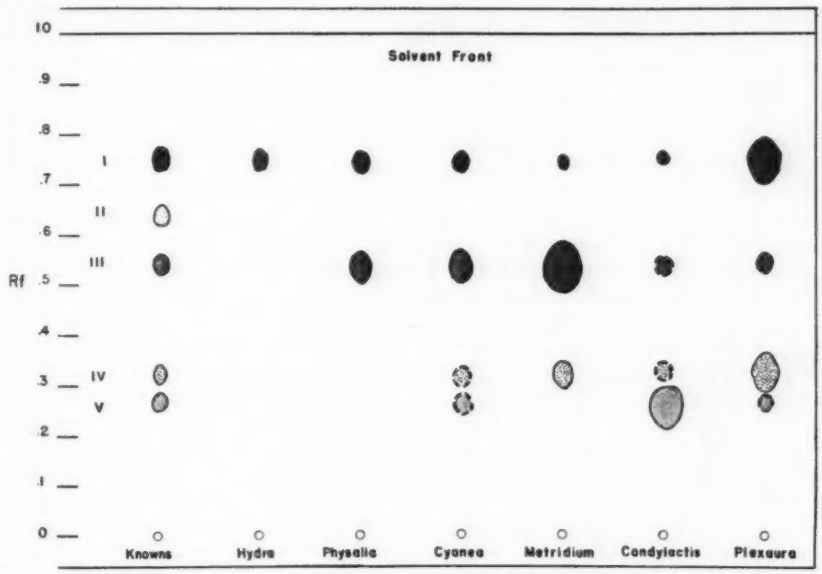

FIGURE 2. Composite of chromatograms giving Rf values for five of the compounds with which this study was concerned. In instances where identification was tentative, the spots are shown with broken boundary lines. I = tetramethylammonium; II = N-methylpyridinium; III = homarine; IV = trigonelline; V = γ-butyrobetaine. Solvent = ethanol-ammonia.

During the period when most of the work reported here was in progress we were not aware of the identification of zoo-anemonin as the dimethylbetaine of imidazole acetic acid. Now having samples of the synthesized material we find what we believe to be zoo-anemonin in *Metridium*, *Condylactis* and *Plexaura*. Unfortunately, when ethanol-ammonia is used as a solvent system, the Rf value of zoo-anemonin is between 0.2 and 0.3. This is so similar to that for γ-butyrobetaine that some other solvent system must be used for their separation. We have tried n-butanol-dioxane-water (4:1:5) as used by Ackermann and Janka (1953) for zoo-anemonin. With this they obtained an Rf of 0.17. Extracts of *Metridium* and

Plexaura run with this solvent give a relatively large spot appearing between Rf 0.1 and 0.2 and probably representing zoo-anemonin.

Identification by ultraviolet absorption

Spots of N-methylpyridinium, trigonelline and homarine are readily detected as absorbing areas when dried, untreated chromatograms are examined with short-

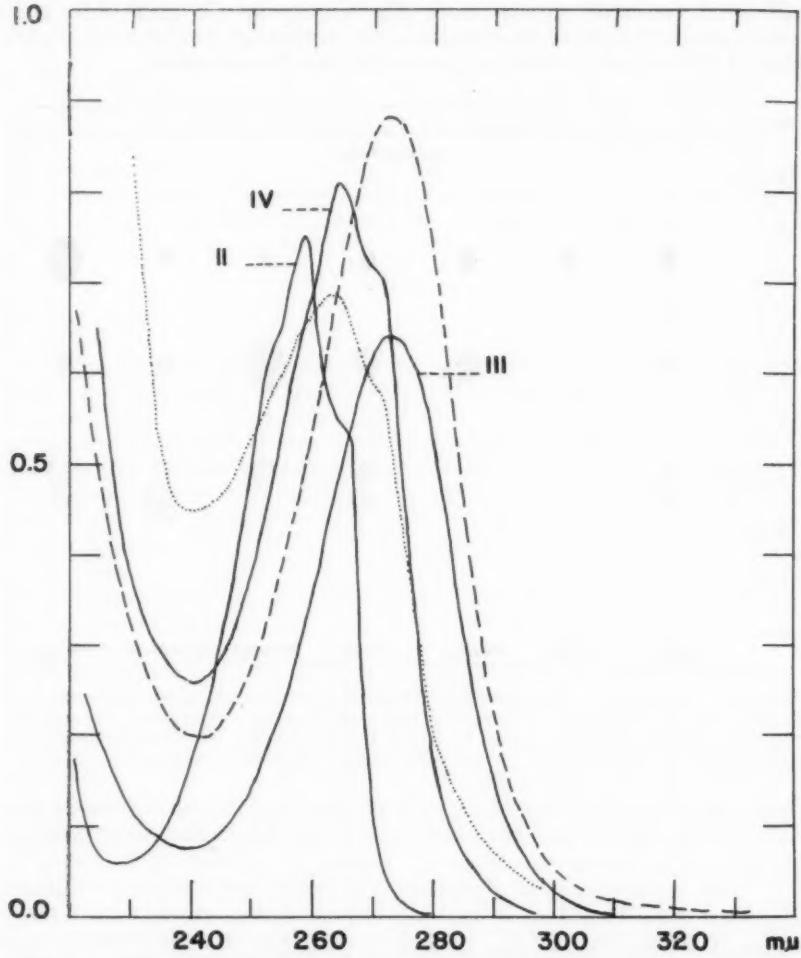

FIGURE 3. Ultraviolet absorption curves for N-methylpyridinium (II), homarine (III), trigonelline (IV), and for eluates of spots from chromatograms of *Metridium* tentacle extracts believed to represent homarine (dashed line) and trigonelline (dotted line). Ordinate = arbitrary units of absorption.

wave ultraviolet light. This was very helpful in the location of regions on the paper where these compounds occurred. Thus, an absorbing area was never found on chromatograms of coelenterate extracts in a region where N-methylpyridinium should have occurred, if it had been present. It should be noted, however, that N-methylpyridinium bromide, when run in ethanol-ammonia, gives two spots, the lower of which is close to homarine. This could give rise to some confusion but we believe, in this instance, that it has not done so.

The ultraviolet-absorbing characteristic of these pyridine derivatives was further used in the identification of homarine and trigonelline. Samples of crystalline N-methylpyridinium, trigonelline and homarine were run in a Cary recording spectrophotometer. Tracings of their absorption curves are combined in Figure 3. Since we were interested mainly in the wave-length at which maximum absorption occurred, their extinction coefficients were not determined. N-methylpyridinium absorbed maximally at 258 mμ, trigonelline at 264 mμ, and homarine at 272–3 mμ.

Extracts of *Metridium* and *Physalia* were streaked on 5-inch-wide strips of paper. After running in ethanol-ammonia the papers were dried and the areas believed to be occupied by trigonelline or homarine were outlined under an ultraviolet light source. These areas were then cut out and eluted with distilled water. After appropriate dilution (determined by trial) the absorption curves for these extracts were also obtained. Figure 3 shows curves for eluates of the areas of *Metridium* chromatograms believed to represent homarine and trigonelline. Maximum absorption of the eluate supposed to contain trigonelline is seen to correspond precisely with that of the authentic samples of trigonelline (IV). The absorption curves for both samples have similar characteristic shoulders.

The absorption curve of a *Metridium* eluate, believed to contain homarine, is identical in shape with that for synthetic homarine. In a similar manner, homarine was identified in extracts of tentacles of *Cyanea* and *Physalia*. The procedure was not used with the other species.

On chromatograms of tentacle extracts of *Metridium* and *Physalia,* run in ethanol-ammonia, an ultraviolet-absorbing area was found with an Rf of about 0.2. Eluates of this area gave absorption curves with a maximum absorption at 248 mμ. The substance responsible for this was not identified.

The action of quaternary ammonium bases on fiddler crabs

Aqueous extracts or homogenates of tentacles of certain coelenterates have been shown to influence the autotomy reflex of crustaceans (Welsh, 1956). Likewise, certain quaternary ammonium bases were found to facilitate or to reduce the tendency to autotomize legs. In order to determine whether or not the bases under investigation in the present study would reproduce the actions of coelenterate extracts, the following experiments were performed. A given volume of *Metridium* tentacles was homogenized with an equal volume of sea water. After centrifuging, 0.05 ml. of the clear supernatant was injected into each of five *Uca pugilator*. In three minutes, six legs had spontaneously autotomized and the crabs were showing signs of severe paralysis, from which none recovered. The original extract was diluted 1:10 with sea water and five crabs injected with 0.05 ml. each. In five minutes, four of the crabs had dropped 25 legs (one crab failed to autotomize any legs). In a few more minutes, they were completely paralyzed and none recovered.

A dilution of 1:100 with sea water produced 12 autotomies in 15 minutes. After 24 hours two crabs were dead and after 48 hours two additional crabs. Thus, an aqueous extract of *Metridium* tentacles contains factors which, in considerable dilution, produce spontaneous autotomy of legs followed by paralysis and death, in this species of crustacean.

An abundance of *Hydra littoralis,* from mass cultures, made it convenient to test the action of a hydra extract on a crustacean. Approximately 2000 hydra, unfed for one week, were blotted and weighed. The wet weight was 410 mg. They were homogenized and one ml. of distilled water added. After centrifuging, 0.02 ml. of the clear supernatant was injected into *Uca pugilator.* The following is a typical record:

1:30 PM	Injected 0.02 ml. at base of second left walking leg of a specimen of *Uca* weighing 3.16 gm.
1:31 PM	First and second left legs "paralyzed"
1:33 PM	Crab cannot right itself when turned on back
1:34 PM	Only slight limb movements
1:40 PM	All spontaneous movements have ceased and no response to stimulation
2:45 PM	No indication of recovery; appears dead, but on removing carapace the heart is found beating.

This extract when diluted 1:10 with sea water was almost as effective in causing paralysis of *Uca* as the undiluted extract. However, when heated at 100° C. for five minutes an injection of 0.02 ml. was entirely without effect on *Uca.*

Next, a series of tests was made to determine whether or not any one of the six bases used in this study, and available in crystalline form, would mimic in any respect the extract of *Metridium* tentacles. Each base was made up as a one per cent solution in sea water and 0.05 ml. injected into each of five *Uca pugilator.* Of the six bases only tetramethylammonium bromide appeared to have significant action. This substance produced a type of paralysis from which only three of five crabs recovered.

It seemed possible that a mixture of the bases in question might have an action that individual members lacked. Therefore, they were combined and injected. The action on the crabs was unspectacular and did not differ from that produced by an equivalent amount of a tetramethylammonium salt. From these injection experiments it would appear that the toxic action of an aqueous extract of *Metridium* tentacles, or whole hydra, on the fiddler crab, *Uca pugilator,* could not be due solely to the presence of the quaternary ammonium bases with which this study was chiefly concerned.

The presence of a tetramethylammonium compound in all species of coelenterates that were examined; its occurrence as the only quaternary base identifiable in *Hydra littoralis* (by the methods used) and its known effects on crustaceans (Welsh, 1956) would all appear to support the earlier suggestion of Ackermann, Holtz and Reinwein (1923) that tetramethylammonium hydroxide (tetramine) might be the paralyzing factor in nematocyst toxin. Two observations made in the present study make this suggestion unlikely. They are (1) that a dose of hydra extract calculated to contain the active material from 0.14 mg. of dry

hydra is fatal to a specimen of *Uca*, while 0.5 mg. of crystalline tetramethylammonium bromide is not, and (2) that heating for 5 minutes at 100° C. destroys or greatly lowers the activity of an aqueous hydra extract. This should have little, if any, effect on a tetramethylammonium salt.

Discussion

Studies made on extracts of whole coelenterates, their tentacles, or their acontia, will not conclusively identify the chemical constituents of nematocyst contents and, therefore, coelenterate or nematocyst toxins, as was recently pointed out by Phillips and Abbott (1957). Such studies may, however, give valuable clues to the nature of the toxic substance, and toxic components of extracts of tissues, rich in nematocysts, may then be sought in extracts of the isolated and cleaned stinging organelles. Methods for isolating undischarged nematocysts have been developed (Phillips, 1956; Phillips and Abbott, 1957) and are being adopted by others (*e.g.* Dodge and Lane, 1958; Lane and Dodge, 1958).

The work reported here was an attempt to learn more about the distribution of tetramine and other quaternary ammonium bases in representative coelenterates. While several bases were found in marine coelenterates, only tetramine was present in the fresh water hydra in sufficient amounts to be identified with the methods employed. This finding, and the observation that tetramine was the only base employed in this study that had significant paralyzing action on *Uca*, provide further evidence that this substance may be a constituent of nematocyst toxin. Almost certainly it is not solely responsible for the paralyzing effects of coelenterate stings. One or more proteins could be additional components. This is suggested by the decreased activity of hydra extracts that have been heated (see above) and by the loss of toxicity by isolated nematocysts that have been treated with ether, alcohol or drying (Phillips and Abbott, 1957). Against the view that proteins may be important in nematocyst toxin is an earlier observation that deproteinization with trichloracetic acid did not significantly alter the toxicity of extracts of acontia of *Adamsia palliata*, when *Carcinus* and *Astacus* were used as test animals (Cantacuzène and Damboviceanu, 1934a). Further observations on the trichloracetic acid extract of *Adamsia* acontia suggest that the crustacean-paralyzing factor is a relatively small and quite stable molecule (Cantacuzène and Damboviceanu, 1934b).

It is not unreasonable to theorize that an association of tetramine with a protein might produce a substance more toxic than tetramine alone. This is based partly on the evidence that two alkylated tetracovalent nitrogens, properly spaced in a molecule, can produce highly active junctional blocking agents such as curare and the many synthetic, curariform, bis-quaternary substances. Protein denaturation by heat, or otherwise, might alter the spacing of the tetramines on the protein or set them free and thereby reduce, but not abolish, the paralyzing action of an extract. In support of such a suggestion is the observation that nematocysts have a high affinity for methylene blue, a basic dye with two methylated nitrogens which, through resonance, may become tetracovalent. This implies that there are molecules within the nematocyst (presumably protein) that bind methylene blue and that might bind other quaternary ammonium bases.

Although certain pyridinium derivatives have a weak curariform action in vertebrates (Craig, 1948) those studied here, as well as the other betaines, were characterized by their lack of paralyzing action on *Uca*. Since homarine, one of the compounds in question, occurs widely in marine invertebrates but not in those from fresh water it has been suggested that it may serve an osmoregulatory function (Gasteiger *et al.*, 1955). This may be the role of some of the other nitrogenous bases of marine invertebrates.

When this study was first begun we tentatively identified one of the bases of *Metridium* and *Physalia* extracts as urocanylcholine (Welsh, 1956). This identification was based partly on the ultraviolet absorption of eluates of chromatograms and their comparison with known urocanylcholine. Although the curves and peaks of absorption correspond rather precisely at a certain pH value, we later learned that the absorption maximum of the eluted material did not change with pH as does that of urocanylcholine (Erspamer and Benati, 1953). Later we learned that the suspected urocanylcholine was actually homarine.

Although we do not yet know what is responsible for the paralysis produced by the nematocysts of coelenterates, the renewed interest in this question should eventually provide an answer.

Summary

This was a study of the identification and distribution of quaternary ammonium bases in representative coelenterates. The purpose was to determine if bases were present with paralyzing actions greater than that of tetramethylammonium (tetramine) which was found to occur in all species examined. Four other bases (homarine, trigonelline, γ-butyrobetaine and the dimethylbetaine of imidazole acetic acid) were found in some species. The bases other than tetramine were found to have no observable paralyzing action on *Uca pugilator*, in the doses employed. However, it is not possible to account for the powerful paralyzing actions of cold, aqueous extracts of *Metridium* tentacles or whole hydra on the basis of their tetramine content. It is suggested that this base, in conjunction with a specific protein, might be responsible for the paralyzing action of nematocysts.

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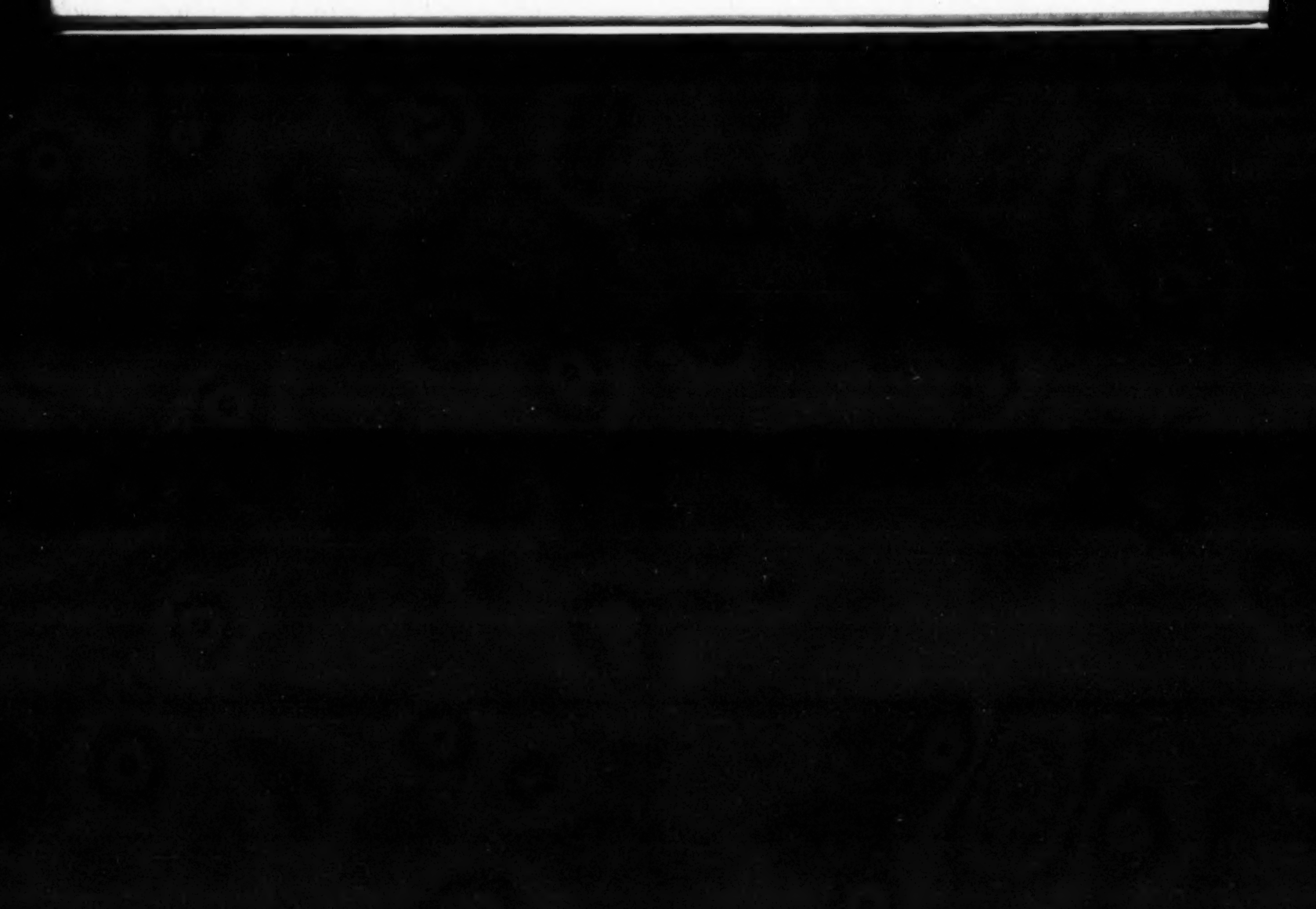

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